#881

Glycom A/S Kogle Allé 4 2970 Hørsholm, Denmark

21 August 2019

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 6'-Sialyllactose Sodium Salt (6'-SL)

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 6'-sialyllactose sodium salt (6'-SL) produced by *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 6'-SL 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,

(b) (6)

Marta H. Mikš, Ph.D., D.Sc.
Regulatory & Scientific Affairs Manager
Glycom A/S

GRAS NOTICE FOR 6'-SIALYLLACTOSE SODIUM SALT (6'-SL)

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:
15 August 2019

GRAS Notice for 6'-Sialyllactose Sodium Salt (6'-SL)

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GRAS Notice for 6'-Sialyllactose Sodium Salt (6'-SL)

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 6'-sialyllactose sodium salt (6'-SL), 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 6'-SL as a food ingredient for addition to non-exempt term infant formula and various conventional food products, as described herein.

Signed,

(b) (6)

21.08.2019

Marta H. Mikš, Ph.D., D.Sc.
Regulatory & Scientific Affairs Manager
Glycom A/S
mhm@glycom.com

Date

1.1 Name and Address of Notifier

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Kogle Allé 4
2970 Hørsholm
Denmark
Tel: +45 2826 3724
Fax: +45 4593 3968

1.2 Common Name of Notified Substance

6'-Sialyllactose sodium salt; 6'-SL

1.3 Conditions of Use

6'-SL is intended to be added to foods targeted to infants and young children, including non-exempt term infant formula, as well as uses in specific conventional food and beverage products used by the general population (Table 1.3-1). Food uses of 6'-SL in infant formula (*i.e.*, infants up to 12 months) will provide 6'-SL at a use-level of 0.4 g/L, follow-on formula at a use-level of 0.3 g/L, infant-specific beverages targeted to young children at a use-level of 0.3 g/L in ready-to-drink and reconstituted products, and up to 2.5 g/kg for products other than beverages (*e.g.*, baby foods). 6'-SL is also intended for use in food and beverages targeted towards the general U.S. population (up to 0.5 g/L or 5 g/kg), and foods for special dietary use

(*e.g.*, meal replacement bars) at levels up to 1.0 g/L or 10 g/kg. The maximum use-levels are proposed on the basis of providing similar levels of 6'-SL on a body weight basis as those consumed by breast-fed infants (see Section 3.1.3). A summary of the food categories and use-levels in which 6'-SL is intended for use is provided in Table 1.3-1 below.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use-Levels for 6'-SL in the U.S.

Food Category (21 CFR §170.3) (U.S. FDA, 2018b)	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	0.24	1.0
	Sports and Isotonic Drinks, Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	360 mL	0.18	0.5
Infant and Toddler Foods	Non-exempt Term Infant Formulas	100 mL[c]	0.04	0.4
	Toddler Formulas	100 mL[c]	0.03	0.3
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.02 to 0.42	2.5
	Other Drinks for Young Children	120 mL	0.04	0.3
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	0.4	10.0
	Cereal and Granola Bars	40 g	0.2	5.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk*	240 mL	0.12	0.5
Milk Products	Buttermilk*	240 mL	0.12	0.5
	Flavored Milk	240 mL	0.12	0.5
	Milk-Based Meal Replacement Beverages, for Weight Reduction[b]	240 mL	0.24	1.0
	Yogurt*	170 g	0.86	5.0

6'-SL = 6'-sialyllactose sodium salt; 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, 2018c). 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.

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

While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of HiMOs, including 6'-SL may be used in combination with other HiMOs such as 3'-SL, 2'-FL, DFL, LNnT, LNT, and future HiMOs that Glycom will develop, to produce infant formula products that are as compositionally representative of human breast milk as possible while taking into account natural variation. The specific combination of HiMOs will be determined by the infant formula manufacturer based on available clinical data and product development goals.

The maximum concentration of Glycom's 6'-SL ingredient that may be added to infant formula is 0.4 g/L and was based on the average of mean values from pooled human breast milk samples from lactating women across all lactational stages. A concentration of 0.4 g/L will provide concentrations of 6'-SL that fall within the 95% percentile mean concentration ranges (See Thurl *et al*., 2017 in Section 3.1.3.2 below) that have been measured in pooled human milk samples, and therefore ensures that infants will consume nutritional quantities of 6'-SL that cover 95% of the population of mothers regardless of secretor status.

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 6'-SL 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

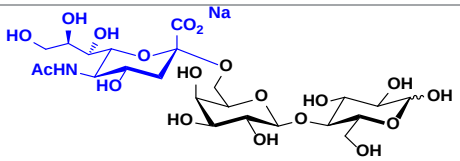
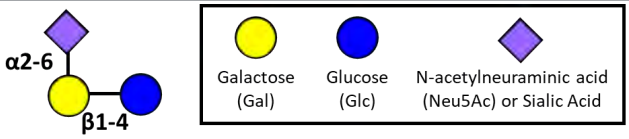
2.1 Identity

6'-SL is predominantly a single purified ingredient, but also contains small quantities of lactose and *N*-acetylneuraminic acid[1] [all 3 are collectively called human-identical milk saccharides (HiMS)[2]]. The final product also contains minor amounts of other related and fully characterized carbohydrates, which are produced during the fermentation process. 6'-SL is a trisaccharide that is derived from lactose by subsequent addition of sialic acid (Neu5Ac) and galactose. Using 1D-^{1}H nuclear magnetic resonance (NMR)-spectroscopy and mass spectrometry, it has been demonstrated that 6'-SL as manufactured by Glycom is chemically and structurally identical to 6'-SL that is naturally present in human breast milk. Further description of the structural and chemical identity of 6'-SL is presented below in Table 2.1-1.

[1] Sialic acid is the more common (but colloquial) synonym for *N*-acetylneuraminic acid (abbreviated either as NANA or Neu5Ac). NANA has GRAS status for use as an ingredient in term infant formula at a maximum use level of 50 mg/L (GRN 602 – U.S. FDA, 2016a).

[2] Human-identical milk saccharides (HiMS) is the sum of 6'-SL, lactose and N-acetylneuraminic acid, which make up the majority of the 6'-SL product. The collective term "HiMS" refers to the fact that all 3 components are naturally occurring components of human breast milk. Please note the term "saccharide" – instead of "oligosaccharide" – is simply due to lactose being a disaccharide (and therefore can't be denoted as an "oligosaccharide").

Table 2.1-1 Identity of 6'-SL

Product Name	6'-Sialyllactose sodium salt	
Abbreviations	6'-SL (6'SL, 6SL, 6-SL)	
IUPAC Name	*N*-Acetyl-α-D-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)- D-glucose, sodium salt	
IUPAC Abbreviation (extended)	α-D-Neu*p*5Ac-(2-6)-β-D-Gal*p*-(1-4)- D-Glc*p*, sodium salt	
IUPAC Abbreviation (condensed)	Neu5Ac-(α2-6)-Gal-(β1-4)-Glc, sodium salt	
Molecular Structure		
Symbol Nomenclature		
Molecular Formula	6'-Sialyllactose 6'-Sialyllactose sodium salt	$C_{23}H_{39}NO_{19}$ $C_{23}H_{38}NO_{19}Na$
Molecular Mass	6'-Sialyllactose 6'-Sialyllactose sodium salt	633.55 g/mol 655.53 g/mol
CAS Number	6'-Sialyllactose 6'-Sialyllactose sodium salt	35890-39-2 157574-76-0
CAS Name	D-Glucose, *O*-(*N*-acetyl-α-neuraminosyl)-(2→6)-*O*-β-D-galactopyranosyl-(1→4)-, sodium salt (1:1)	
Synonyms	6'-*O*-(*N*-Acetylneuraminyl)-lactose, α2,6-sialyllactose	

6'-SL = 6'-sialyllactose sodium salt, CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry.

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, *Escherichia 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, which has been used as a host strain for further development of the 3'-SL production strain was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection (deposited under DSM No. 4235)[3]. *E. coli* K-12 and its derivatives[4] 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

[3] www.dsmz.de

[4] 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).

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'-fucosyllactose (2'-FL; (U.S. FDA, 2016b, 2018d,e), lacto-*N*-neotetraose (LNnT; U.S. FDA, 2016c), alpha cyclodextrin (U.S. FDA, 2004), chymosin (U.S. FDA, 2018f), L-leucine (U.S. FDA, 2010), and β-galactosidase (U.S. FDA, 2014)].

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

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

2.2.1.2 Production Strain

The host strain *E. coli* K-12 DH1 (DSMZ, 2015) was optimized for general oligosaccharide expression features (used as a "platform host strain") by introduction of 7 modifications related to the metabolism of various carbohydrates, thereby improving the efficiency of the strain. This strain was given the designation "MDO". An overview of the modification events used for construction of strain MDO has been discussed previously and is hereby incorporated by reference to Section II.B.1.2 of GRAS Notice (GRN) 650. The genetic modifications applied to the platform and production strains were verified by applying whole genome sequencing (at the level of MDO strain, Steps 1 to 7) and colony polymerase chain reaction (PCR) targeted sequencing methods. This parental strain has served as the host for engineering all of Glycom's production strains that are used to produce other HiMOs that have GRAS status, including LNnT, 2'-FL, 2'-FL/difucosyllactose (DFL), and LNT (U.S. FDA, 2016b,c, 2018e).

The MDO platform strain was further modified to generate the production strain, *E. coli* (K-12 DH1 MDO) MAP265, to biosynthesize 6'-SL by targeted insertion of one or more copies of a truncated bacterial α-2,6-sialyltransferase, along with targeted insertion of a bacterial derived gene cluster encoding enzymes for the synthesis of activated sialic acid (α-N-acetylglucosame-6-phosphate epimerase, sialic acid synthase, and CMP-Neu5Ac). The gene cluster involved in NAD biosynthesis was disrupted and re-introduced into a plasmid to tightly link cell survival to 6'-SL production; this plasmid also contained an additional copy of the same bacterial gene cluster enabling synthesis activated sialic acid that was introduced into the chromosome. The biochemical pathway by which the production strain generates 6'-SL using D-lactose (substrate) and D-glucose[5] (carbon source) is shown in Figure 2.2.1.2-1 below.

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

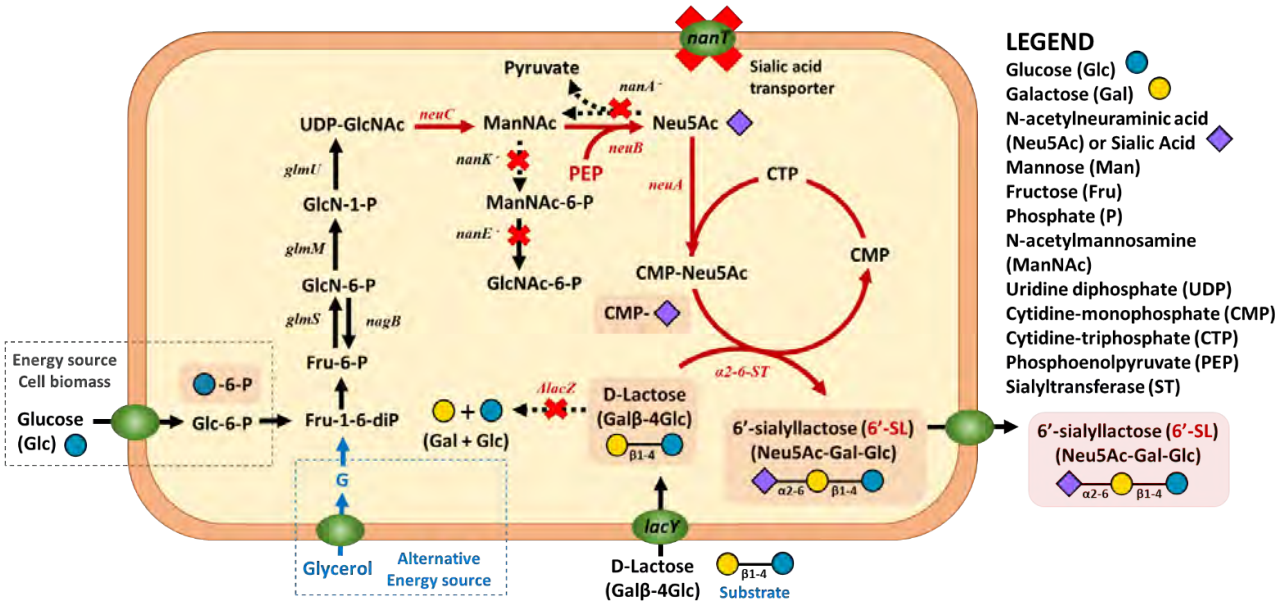
The production strain is a stable and reliable microorganism that provides high titers of 6'-SL. The strain has been deposited in the DSMZ in Braunschweig, Germany, under DSM No. 32833. Defined DNA sequences from the donor microorganisms were identified using genome databanks, codon-optimized by bioinformatic tools (when needed), extended with appropriate restriction enzyme recognition sequences to allow directed cloning and then synthesized by DNA synthesis, and are therefore referred to as "synthetic DNA"[6]. The gene cassettes used for introduction of the donor genes to the organism are well-characterized and have been sequenced to verify their identities. As the introduced genes are produced by DNA synthesis from well-characterized annotated genomes and were not cloned directly from the host genome using PCR based methods there is no risk of introducing unintended and undesirable genes from the donor organisms to the production organism.

The inserted enzyme is well-characterized and based on its enzymatic function, it was concluded that the introduced gene would not confer toxicogenic or pathogenic properties to the host organism. The genetic modifications made to the production strain result in the expression of proteins that are involved in the normal carbohydrate processing within their donor sources. Bioinformatic searches conducted using the amino acid sequences of the proteins introduced to the *E. coli* K-12 (DH1) MAP265 strain by genetic modification confirmed that there is no relevant homology to known protein toxins or to known allergens. The genetic modifications applied to the platform and production strains were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods.

The production strain has a plasmid, however, lacks the presence of antibiotic resistance genes. In addition, throughout the fermentation process of 6'-SL the use of antibiotics or inducer molecules are not employed. During manufacture, the production strain remains intact, secretes the 6'-SL 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.

[6] No synthetic DNA sequences were used in the sense of artificial, rationally *de novo* designed sequences. All DNA sequences were identified in the databanks of naturally occurring and annotated genes. Certain selected sequences were codon optimized; however, they continued to encode the same protein sequence after DNA optimization.

Figure 2.2.1.2-1 Pathway for 6'-SL Biosynthesis by the *Escherichia coli* K-12 DH1 MDO-derived 6'-SL Production Strain



6'-SL = 6'-sialyllactose sodium salt.

2.2.2 Description of the Production Process

Glycom's 6'-SL is manufactured in compliance with cGMP and the principles of Hazard Analysis Critical Control Point (HACCP). The manufacture of 6'-SL 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, 2016b,c). All additives, processing aids, and food contact articles used during manufacturing are permitted by federal regulation, have been previously determined to be GRAS for their respective uses, or have been the subject of an effective food contact notification. The manufacturing process can be broadly divided into 2 stages.

In Stage 1 [upstream processing (USP)], D-lactose and D-glucose are converted to 6'-SL by the adapted cellular metabolism of the 6'-SL production microorganism, which uses D-glucose[7] as a carbon source and D-lactose as a substrate for 6'-SL 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 6'-SL product.

A schematic overview of the manufacturing process for 6'-SL is presented in Table 2.2.2-1 below.

[7] D-Glycerol and D-sucrose are alternative carbon sources to D-glucose.

Table 2.2.2-1 Overview of the Manufacturing Process for the 6'-SL Product

STAGE 1		Upstream Processing (USP)
STEPS	1	Media Preparation
	2	Propagation
	3	Seed Fermentation
	4	Fermentation Phases:
	4A	Growth (Batch) Phase[a]
	4B	Feeding (Fed-Batch) Phase
	5	Removal of Microorganism *

STAGE 2		Downstream Processing (DSP)
STEPS	6	Purification/Concentration 1 *
	7	Decolorization *
	8	Ion Removal
	8A	Anion Exchange
	8B	Cation Exchange
	9	Purification/Concentration 2 *
	10	Drying
	11	Sampling and Packaging
	12	Quality Control and Batch Release

6'-SL = 6'-sialyllactose sodium salt.
[a] The batch phase of fermentation is optional.
* After the marked steps additional sterile filtration (microfiltration) is performed to maintain low microbial load during all times of downstream processing and to ensure high microbial quality of the final ingredient. These steps are further reassurance of absence of the production microorganism in final ingredient.

2.2.3 Quality Control

6'-SL is manufactured in compliance with applicable provisions of the Food, Drug, and Cosmetic Act, Food Safety Modernization Act, and regulations implemented thereunder, including those related to hazard analysis and risk-based preventive controls.

Considering the chemically well-characterized principal raw materials and final products, the production process as a whole can be followed in detail by a range of analytical techniques. These techniques are applied either as in-process controls or at batch release (by Certificate of Analysis) to allow full control of the production process (refer to Table 2.2.2-1).

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

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

Incorporation of sterile filtration units throughout the manufacturing process of the HiMOs, ensures high microbiological purity while the production microorganism is not detectable in the final product. The product microorganism is efficiently removed in the ultrafiltration step, which is applied directly following fermentation. In addition, several additional purification steps are carried out in the down-stream processing stage to help achieve a highly purified 6'-SL, which is free from bacterial cells and residual fermentation by-products. The absence of the microorganisms can be measured by analysis for *Enterobacteriaceae* in the final product according to internationally recognized methods (ISO21528-1:2004; MSZ ISO 21528-2:2004). The ISO 21528-1:2004 method involved a pre-enrichment step that is specifically applicable to microorganisms expected to need resuscitation prior to enrichment.

E. coli K-12 (a gram-negative bacterium) possesses complex glycolipids of high molecular weight in their cell membrane and are known as lipopolysaccharides (LPS). When LPS enters the blood stream, it is recognized by immune cells and an immune response is elicited, which can result in a serious effect in infusion therapy and parenteral nutrition. LPS is also referred to as an endotoxin; however, this is not to be confused with protein-type toxins; however, LPS from *E. coli*-K12 lacks O-antigen epitopes that are characteristic of pathogenic strains of *E. coli*. Following ingestion of LPS, no adverse effects are observed, and this is likely due to a combination of deactivation from stomach acid and a low absorption due to high molecular weight. Moreover, since LPS is an intrinsic component of the cell membrane of gram-negative bacteria, background exposure of the gastrointestinal tract to LPS from the resident microbiota is high. A strict specification for endotoxins is set to control for potential residual endotoxin levels to confirm the high purity of the product in terms of endotoxins.

The absence of traces of residual DNA in the product following fermentation and purification of 6'-SL is confirmed by 3 different validated quantitative PCR (qPCR) methods. These methods target short subsequences of the inserted genes encoding sialic acid synthase genes and α-2,6-sialyltransferase[8] as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. All 3 methods are validated to detect traces of DNA down to 4 pg/mg (parts per billion). The qPCR tests were applied to all analyzed batches and the results were below the limit of quantification (LOQ) in all tested batches (see Table 2.3.3.3-1).

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

The specifications for 6'-SL are presented in Table 2.3.1-1. The parameters include specifications for 6'-SL and the total HiMS content (*i.e.,* 6'-SL, lactose, and sialic acid). Limits for fermentation carry-over products (*e.g.,* sodium and chloride) and possible degradation[9] products such as 6'-sialyl-lactulose have been established. Analysis of the HiMS content of 6'-SL is conducted using high-performance anion exchange chromatograph (HPAEC) coupled with pulsed amperometric detection (PAD) analysis. Upper limits have also been established for microbiological parameters. Two specification limits have been established for microbiological parameters depending on whether the 6'-FL ingredient is utilized in wet-blending or dry-blending of the infant formula production process. The wet-blending stage of manufacturing involves a heat-treatment step in which these microorganisms would be killed. Heat-treatments at temperatures above 75°C for 30 seconds will provide a reduction in excess of 10 log units of vegetative microorganisms such as *Salmonella* spp. or *Enterobacteriaceae*, including *C. sakazakii*; heat-treatments above 100°C will lead to reductions in excess of several hundred log units (WHO, 2006). Microbial specifications used for wet-

[8] Including its recombinant truncated form.
[9] Please note that lactose and sialic acid are also principal degradation products of 6'-SL.

blending applications will therefore be compliant with the microbial requirements for infant formula as defined under 21 CFR §106.55.

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 6'-SL

Definition					
6'-Sialyllactose sodium salt (6'-SL) 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 (6'-SL)	RT of standard ± 3%	Complies			Glycom method HPAEC-HMO-009
Assay (water free) – Sum of HiMS[a]	Not less than 94.0 w/w %	98	±	1	Glycom method HPAEC-HMO-009 and -010
Assay (water free) – 6'- SL	Not less than 90.0 w/w %	95	±	1	Glycom method HPAEC-HMO-009
D-Lactose	Not more than 5.0 w/w %	2.2	±	0.4	Glycom method HPAEC-HMO-010
Sialic acid	Not more than 2.0 w/w %	0.6	±	0.2	Glycom method HPAEC-HMO-009
6'-Sialyl-lactulose	Not more than 3.0 w/w %	0.7	±	0.1	Glycom method HPAEC-HMO-009
Sum of other carbohydrates	Not more than 3.0 w/w %	0.6	±	0.1	Glycom method HPAEC-HMO-009 and -010
pH (20°C, 5% solution)	4.5 to 6.0	5.5	±	0.2	Ph. Eur. 9.2 2.2.3 (07/2016:20203)
Water	Not more than 6.0 w/w %	1.1	±	0.1	Glycom method KF-002
Assay sodium	2.5 to 4.5 w/w %	3.2	±	0.1	EN 13805:2002; EPA-6010C:2007
Chloride by IC	Not more than 1.0 w/w %	0.05	±	0.05	ISO 10304-2:1999
Residual protein by Bradford assay	Not more than 0.01 w/w %	< 0.0017			Glycom method UV-003
Lead	Not more than 0.1 mg/kg	<0.1			EN 13805:2002; EPA-6020A:2007
Microbiological Parameters[b]					
Aerobic mesophilic total plate count	Not more than 1000 CFU/g	< 10			ISO 4833-1:2014
Enterobacteriaceae	Not more than 10 CFU/g	Complies			ISO 21528-1:2004, ISO 21528-2:2004
Salmonella spp.	Absent in 25 g	Complies			ISO 6579:2006
Yeasts	Not more than 100 CFU/g	< 10			ISO 7954:1999
Molds	Not more than 100 CFU/g	< 10			ISO 7954:1999
Residual endotoxins	Not more than 10 EU/mg	0.32	±	0.32	Eur. Ph. 2.6.14

6'-SL = 6'-sialyllactose sodium salt; AVE = average; CFU = colony-forming units; EPA = Environmental Protection Agency; EU = endotoxin units; Eur Ph. = European Pharmacopoeia; HiMS = Human-identical milk saccharides; HPAEC = high-performance anion exchange chromatography; IC = ion chromatography; ICP = inductively coupled plasma; KF = Karl-Fischer; SD = standard deviation; RT= retention time.

[a] HiMS = Sum of 6'-SL, lactose, and sialic acid.

[b] The microbial specifications listed represent minimum requirements for 3'-SL that is added to infant formula and toddler formula products during the wet-mix stage of the infant formula manufacturing process, and also is suitable for conventional food

Table 2.3.1-1 Specifications for 6'-SL

products used by the general population (*i.e.,* non-infant formula and toddler formula food products). The minimum microbial requirements for 3'-SL that is added during the dry-blending stage of infant formula manufacturing include the following additional parameters: *Cronobacter (Enterobacter) sakazakii* (Absent in 10 g), *Listeria monocytogenes* (Absent in 25 g), and *Bacillus cereus* (not more than 50 CFU/g).

2.3.2 Product Analyses

2.3.2.1 Main products and Other Carbohydrates

The physicochemical properties of 6'-SL as manufactured by Glycom can be described as white to off-white amorphous powder or agglomerate. Amorphous powders do not possess defined melting points. 6'-SL is readily soluble in aqueous solutions (maximum 500 mg/mL, 25°C), with poor solubility in any organic solvents. The summary of batch results corresponding to selected physio-chemical properties of 6'-SL ingredient is presented in Table 2.3.2.1-1.

Table 2.3.2.1-1 Batch Results for Selected Physicochemical Properties of 6'-SL Product

Parameters	**Manufacturing Batch Numbers:**							
	CPN5315 1000117 FD	**CPN5315 1000317 FD**	**CPN5315 1000417 FD**	**CPN5315 1000617 FD**	**CPN5315 1000717 FD**	**AVE**	**±**	**SD**
Appearance	Powder or agglomerates							
Color	White to off-white							
pH (20°C, 5% solution)	5.4	5.2	5.3	5.6	5.8	**5.5**	**±**	**0.2**

6'-SL = 6'-sialyllactose sodium salt; AVE = average; SD = standard deviation.

6'-SL is a purified carbohydrate ingredient (purity *ca.* 95%) which contains minor amounts of related and fully characterized carbohydrates (*e.g.,* lactose, sialic acid, 6'-sialyllactulose). The ingredient predominately consists of lactose (≤5.0%) and sialic acid (≤2.0%) which are naturally occurring compounds that are found in human milk. The results of the HPAEC-PAD analyses are shown in Table 2.3.2.1-2 below.

Collectively, the HiMS fraction (6'-SL, lactose and sialic acid) of the product comprises, on average, 98% of the total batch weight. The remaining portion of the product is made up of other carbohydrate-type compounds that are structurally related to 6'-SL, for example 6'-sialyl-lactulose. Overall, the water-free total specified carbohydrates fraction of the 6'-SL ingredient accounts for over 99% of the final batch weight.

Table 2.3.2.1-2 Batch Results for Fermentation Metabolites and Other Carbohydrates By-Products for 6'-SL

Parameters	Manufacturing Batch Numbers:							
	CPN5315 1000117 FD	CPN5315 1000317 FD	CPN5315 1000417 FD	CPN5315 1000617 FD	CPN5315 1000717 FD	AVE	±	SD
Assay (water free) – Sum of HiMS [a][%]	98.0	99.9	99.4	96.6	98.1	**98.4**	±	**1.3**
Assay (water free) – 6'-SL	94.3	96.8	97.1	93.9	95.3	**95.5**	±	**1.4**
Sialic Acid	0.68	0.51	0.33	0.81	0.85	**0.64**	±	**0.22**
D-Lactose [%]	2.89	2.52	2.01	1.92	1.94	**2.26**	±	**0.43**
6'-Sialyl-lactulose [%]	0.54	0.75	0.76	0.65	0.72	**0.68**	±	**0.09**
Sum of other carbohydrates [%]	0.56	0.55	0.42	0.63	0.66	**0.6**	±	**0.1**
Total specified carbohydrates (water free) [%]	99.0	101.1	100.6	97.9	99.5	**99.6**	±	**1.3**

6'-SL = 6'-sialyllactose sodium salt; AVE = average; HiMS = human-identical milk saccharides; SD = standard deviation.
[a] Sum of HiMS = Sum of 6'-SL, lactose, and sialic acid.

6'-SL is an acidic trisaccharide that is naturally present at significant amounts in human milk. 6'-SL is the most abundant acidic sialylated HMO found in human milk (*ca.* 0.4 to 0.5 g/L) and colostrum, with wide ranges reported between mothers (0.1 to 1.3 g/L).

6'-SL is comprised of *N*-acetylneuraminic acid (or sialic acid), D-galactose, and D-glucose. Alternatively, the molecular constitution can be described as consisting of the monosaccharide sialic acid and the disaccharide D-lactose, which are linked by a α(2→6) bond to form the trisaccharide. 6'-SL is a chemically defined trisaccharide and the constitutional isomer of another acidic sialylated HMO, 3'-sialyllactose sodium salt (3'-SL). Both HMOs, 6'-SL and 3'-SL, are naturally present in human and other mammalian milks (Yan *et al.,* 2018), and are reported to be the predominant milk oligosaccharides in some species such as dogs' milk where levels of 0.6 g/L and 7.5 g/L have been measured for 3'-SL and 6'-SL, respectively (Macias Rostami *et al.,* 2014). The HiMO 6'-SL, obtained from microbial fermentation, is chemically and structurally identical to 6'-SL that is naturally present in human breast milk (HMO 6'-SL), as confirmed by ^{1}H- and 2D-NMR-spectroscopy, and mass spectrometry.

The remaining saccharides lactose and sialic acid are natural components of human breast milk and the resulting exposure from their levels as components of 6'-SL would be insignificant compared to the exposure from each of these saccharides at their naturally occurring levels. Lactose is known to be the most abundant single molecule of milk, while sialic acid is commonly present (in bound or free form) in human body and its fluids (Wang *et al.*, 2001; Röhrig *et al.*, 2017). The average total sialic acid content (free and bound) of human milk is high, with levels ranging between 900 and 1,800 mg/L in early milk (colostrum, transition milk, and first month milk; see Section IV.B.1 of GRN 602).

6'-Sialyl-lactulose is an isomer of 6'-SL, arising from the isomerization of the terminal glucose moiety of 6'-SL 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 relative levels between

1 and 7% of their lactose content, and absolute levels up to 13.7 mmol/L (Beach and Menzies, 1983). Although the isomerization product of 6'-SL 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 6'-sialyl-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. 6'-sialyl-lactulose is not absorbed intact and microbial fermentation will be indiscriminate of the lactose isomer type producing the same innocuous products of glucose and galactose fermentation [*i.e*., short-chain fatty acids (SCFA), CO_2, H_2]. In any case, at the low levels of this isomerization product (not more than 1.0%) as in presented 6'-SL batches it is expected to be negligible and not biologically/nutritionally relevant.

2.3.2.2 Non-carbohydrate Residues

All batches were tested for residual water and sodium and chloride content, since 6'-SL is produced as a sodium salt. The sodium content of pure 6'-SL, a mono-sodium salt, is at a theoretical concentration of 3.5% compared to the average value of 3.2% measured (considering standard deviations, and the actual 6'-SL assay value that is on average 95.5% and thus leads to a calculated sodium content of 3.3%). The results for the water content of 6'-SL are summarized below in Table 2.3.2.2-1.

Table 2.3.2.2-1 Batch Results for Non-Carbohydrate Residues of 6'-SL

Parameter	Manufacturing Batch Numbers:							
	CPN5315 1000117 FD	CPN5315 1000317 FD	CPN5315 1000417 FD	CPN5315 1000617 FD	CPN5315 1000717 FD	AVE	±	SD
Water [%]	0.38	0.44	0.42	0.44	0.47	**0.43**	**±**	**0.03**
Sodium (Na) [%]	3.27	3.23	3.06	3.15	3.25	**3.19**	**±**	**0.09**
Chloride by IC [%]	0.130	< 0.003	< 0.002	0.049	0.079	**0.053**	**±**	**0.054**

6'-SL = 6'-sialyllactose sodium salt; AVE = average; IC = ion chromatography; SD = standard deviation.

2.3.2.3 Microbial Contaminants

The microbiological purity of 6'-SL production batches has been assessed for non-pathogenic microorganisms (bacteria, yeasts, and molds) as general hygiene indicators, and as well for selected food-borne pathogens and is summarized in Table 2.3.2.3-1 below.

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 6'-SL ingredient, respectively. The 6'-SL ingredient was also tested for the absence of pathogenic bacteria, namely *Salmonella* spp., *Cronobacter sakazakii* and *Listeria monocytogenes*. Spore-forming bacteria *Bacillus cereus*, which is a frequent contaminator of heat-treated or spray-dried foods, was also measured to control the number of surviving spores in the final product.

Table 2.3.2.3-1 Batch Results for Microbiological Analysis of 6'-SL

Microbiological Parameters	Manufacturing Batch Numbers:							
	CPN5315 1000117 FD	CPN5315 1000317 FD	CPN5315 1000417 FD	CPN5315 1000617 FD	CPN5315 1000717 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**		

Table 2.3.2.3-1 Batch Results for Microbiological Analysis of 6'-SL

Microbiological Parameters	Manufacturing Batch Numbers:					
	CPN5315 1000117 FD	CPN5315 1000317 FD	CPN5315 1000417 FD	CPN5315 1000617 FD	CPN5315 1000717 FD	AVE ± SD
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**
Yeasts [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**
Molds [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**

6'-SL = 6'-sialyllactose sodium salt; AVE = average; CFU = colony-forming units; SD = standard deviation.

2.3.3 Manufacturing By-Products, Impurities and Contaminants

Internal quality control measures have been established to include testing for carbohydrate-type metabolites and potential residual compounds and trace elements. These include analyses for amino acids and biogenic amines, microbial endotoxins, 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

6'-SL is 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 6'-SL 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 in their cell walls, called either LPS or endotoxins (not to be confused with protein-type toxins). Internal specifications for endotoxin have been established [max. 10 endotoxin units (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 in the 6'-SL produced by fermentation, was assayed using the *Limulus* amoebocyte lysate kinetic chromogenic assay. In addition, a sensitive residual protein test, based on the Bradford assay, has been developed and applied. Because batch analyses of 6'-SL product demonstrated low endotoxin and residual protein concentrations, they were not considered as compositional or safety-related data of the 6'-SL 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 to identify process deviations in a sensitive manner (see Table 2.3.1-1).

Table 2.3.3.2-1 Batch Results for Microbial Endotoxins and Residual Proteins in 6'-SL

Parameters	Manufacturing Batch Numbers:					
	CPN5315 1000117 FD	CPN5315 1000317 FD	CPN5315 1000417 FD	CPN5315 1000617 FD	CPN5315 1000717 FD	AVE ± SD
Residual endotoxins [EU/mg]	0.01	0.31	0.34	0.10	0.84	**0.32 ± 0.32**
Residual protein by Bradford assay [%]	< LoR[a]	< LoR	< LoR	< LoR	0.005	**< 0.005**

6'-SL = 6'-sialyllactose sodium salt; 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 during USP, which is applied directly after the fermentation. During DSP, sequential filtration (including microfiltration to achieve retention of microorganisms and thus high microbial quality) and purification processes are carried out to ensure a high-purity 6'-SL. The final product is tested for bacteria of the *Enterobacteriaceae* family according to internationally recognized methods (ISO 21528-1:2004, MSZ ISO 21528-2:2004) to ensure the absence of production microorganisms. A pre-enrichment step, recognized by ISO 21528-1:2004, is also carried out to allow for the resuscitation of the microorganism before enrichment and enumeration.

Analyses for residual DNA are also carried out to corroborate the absence of the production organism in final production batches. Three different validated qPCR methods were carried out to confirm the absence of residual DNA from the production organism and is summarized in Table 2.3.3.3-1 below. Short subsequences of the inserted genes as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli* are targeted in the qPCR methods. Based on the analysis of 5 batches of 6'-SL, no detectable levels of residual DNA (limit of quantification of 4 µg/kg or 4 ppb) were observed in the final product.

Table 2.3.3.3-1 Levels of Residual DNA in 5 Batches of 6'-SL

Parameter	Manufacturing Batch Numbers:					
	CPN5315 1000117 FD	CPN5315 1000317 FD	CPN5315 1000417 FD	CPN5315 1000617 FD	CPN5315 1000717 FD	AVE
Residual DNA by qPCR (neuBCA assay)	< LoQ[a]	< LoQ	< LoQ	< LoQ	< LoQ	**< LoQ**
Residual DNA by qPCR (Pd2 assay)	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	**< LoQ**
Residual DNA by qPCR (23S assay)	< LoQ	< LoQ	< LoQ	< LoQ	< LoQ	**< LoQ**

6'-SL = 6'-sialyllactose sodium salt; AVE = average; DNA = deoxyribonucleic acid; neuBCA = sialic acid synthase; LoQ = limit of quantitation; Pd2 = sialyltransferase gene; 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

Trace levels of elements and minerals may be present in the 6'-SL ingredient as a result of the fermentation process (carry-over from the fermentation medium). Through nanofiltration and ion-exchange purification techniques, carry-over of minerals from fermentation into the final ingredient is significantly reduced to negligible levels and are thus not proposed to be added to the specifications of 6'-SL. Analysis of the trace elements are summarized below in Table 2.3.3.4-1. In addition, measurements of trace elements include confirmation of the absence of toxic heavy metals (such as lead).

Table 2.3.3.4-1 Levels of Anions, Trace Elements and Heavy Metals in 5 Batches of 6'-SL

Parameter	Manufacturing Batch Numbers:							
	CPN5315 1000117 FD	CPN5315 1000317 FD	CPN5315 1000417 FD	CPN5315 1000617 FD	CPN5315 1000717 FD	AVE	±	SD
Orthophosphate by UV [%]	< 0.0002	< 0.0003	< 0.0004	0.0003	< 0.0003	**0.0003**	±	**0.0001**
Sulphate by IC [%]	< 0.010	< 0.010	< 0.009	< 0.010	< 0.010	**< 0.010**		
Citrate [%]	< LoD	< LoD	< LoD	< LoD	< LoD	**< 0.020**		
Ammonium by UV [%]	0.00035	0.00021	0.00009	0.00026	0.00032	**0.00025**	±	**0.0001**
Chloride by IC [%]	0.130	< 0.003	< 0.002	0.049	0.079	**0.053**	±	**0.054**
Potassium (K) [mg/kg]	330	120	170	150	160	**186**	±	**83**
Magnesium (Mg) [mg/kg]	< 10	< 10	< 10	< 10	< 10	**< 10**		
Iron (Fe) [mg/kg]	3.0	6.0	3.0	3.0	2.0	**3.4**	±	**1.5**
Zinc (Zn) [mg/kg]	2.9	0.2	0.3	0.3	0.4	**0.8**	±	**1.2**
Copper (Cu) [mg/kg]	< 0.1	< 0.1	< 0.1	< 0.1	0.1	**< 0.1**		
Manganese (Mn) [mg/kg]	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	**< 1.0**		
Lead (Pb) [mg/kg]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	**< 0.1**		

6'-SL = 6'-sialyllactose sodium salt; AVE = average; IC = ion chromatography; LoD = limit of detection; SD = standard deviation; UV = ultraviolet.

2.4 Stability

Storage (real-time and accelerated) and stressed (forced) stability studies on the pure ("bulk") powdered 6'-SL have been conducted by Wessling (Hungary) in accordance to 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:

1. Test the 6'-SL stability during storage;
2. Investigate degradation pathways when exposed to selected stress factors; and
3. Define the optimal storage conditions and corresponding re-test dates or shelf-lives.

For the bulk 6'-SL ingredient, experiments were performed in solid state (in form of amorphous powder) and in liquid form (as aqueous solutions). In addition, the accelerated stability study for the bulk 6'-SL ingredient in solid state has been presented.

Stability studies in processed foods included new studies of 6'-SL in powdered infant formula.

2.4.1 Bulk Stability

2.4.1.1 Real-Time Stability

The bulk stability of 6'-SL produced from fermentation, as described herein, was investigated under real-time conditions [25°C, 60% relative humidity (RH)]. The chemical, physical, microbiological, and sensory testing has been performed in an ongoing 5-year storage study (25°C, 60% RH) on 2 representative batches (No. CPN5315 1000317 FD and CPN5315 1000617 FD), with interim results available up to 12 months (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 12 months.

Table 2.4.1.1-1 Results of the 5-Year Real-Time Stability Study on 6'-SL Ingredient (25°C, 60% Relative Humidity, RH) for 2 Representative Batches: A) Batch No. CPN5315 1000317 FD, and B) Batch No. CPN5315 1000617 FD

A) Manufacturing Batch Number CPN5315 1000317 FD

Parameter	Sample Time (Months)				
	0	3	6	9	12
Physical Properties					
Appearance	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated material, crystalline in appearance
Color	White	White	White	White	White
Purity					
Assay (water free) HiMS [%]	101.3	99.5	100.6	99.5	100.2
Assay (water free) – 6'-SL [%]	97.8	96.1	97.2	96.2	96.8
Lactose [%]	2.80	2.84	2.76	2.80	2.90
Sialic acid [%]	0.59	0.49	0.51	0.48	0.54
6`-Sialyl-lactulose [%]	0.62	0.80	0.78	0.78	0.85
Largest unspecified impurity [%]	0.00	0.00	0.00	0.00	0.00
Unspecified impurities [%]	0.00	0.00	0.00	0.00	0.00
Water content [%]	1.72	1.85	1.70	1.44	1.59
Microbiological Quality					
Aerobic mesophilic total plate count [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10
Enterobacteriaceae	Not tested	Not tested	Not tested	Not tested	Absent in 10 g
Salmonella spp.	Not tested	Not tested	Not tested	Not tested	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Not tested	Not tested	Not tested	Not tested	Absent in 10 g
Listeria monocytogenes	Not tested	Not tested	Not tested	Not tested	Absent in 25 g
Bacillus cereus [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10
Yeasts [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10
Molds [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10

6'-SL = 6'-sialyllactose sodium salt; CFU = colony-forming units; HiMS = human-identical milk saccharides = Sum of 6'-SL, lactose and sialic acid.

Table 2.4.1.1-1 Results of the 5-Year Real-Time Stability Study on 6'-SL Ingredient (25°C, 60% Relative Humidity, RH) for 2 Representative Batches: A) Batch No. CPN5315 1000317 FD, and B) Batch No. CPN5315 1000617 FD

B) Manufacturing Batch Number CPN5315 1000617 FD

Parameter	Sample Time (Months)				
	0	3	6	9	12
Physical Properties					
Appearance	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated material, crystalline in appearance
Color	White	White	White	White	White
Purity					
Assay (water free) HiMS [%]	97.8	97.7	100.1	100.1	99.2
Assay (water free) – 6'-SL [%]	94.4	94.4	96.7	96.9	95.8
Lactose [%]	2.49	2.50	2.50	2.45	2.56
Sialic acid [%]	0.89	0.76	0.79	0.74	0.79
6`-Sialyl-lactulose [%]	0.59	0.79	0.75	0.78	0.87
Largest unspecified impurity [%]	0.00	0.00	0.00	0.00	0.00
Unspecified impurities [%]	0.00	0.00	0.00	0.00	0.00
Water content [%]	1.50	1.79	1.50	1.30	1.59
Microbiological Quality					
Aerobic mesophilic total plate count [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10
Enterobacteriaceae	Not tested	Not tested	Not tested	Not tested	Absent in 10 g
Salmonella spp.	Not tested	Not tested	Not tested	Not tested	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Not tested	Not tested	Not tested	Not tested	Absent in 10 g
Listeria monocytogenes	Not tested	Not tested	Not tested	Not tested	Absent in 25 g
Bacillus cereus [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10
Yeasts [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10
Molds [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10

6'-SL = 6'-sialyllactose sodium salt; CFU = colony-forming units; HiMS = human-identical milk saccharides = Sum of 6'-SL, lactose and sialic acid.

2.4.1.2 Accelerated Stability

The bulk stability of spray dried, amorphous 6'-SL ingredients has been investigated under accelerated conditions (40°C, 75% RH) for a period of 2 years. The results for 2 representative batches No. CPN5315 1000317 FD and CPN5315 1000617 FD are presented in Table 2.4.1.2-1 below. The results of these studies indicate that there are no changes in organoleptic properties of 6'-SL, no appreciable degradation of 6'-SL, no changes in impurity profile, and no alterations in the microbiological quality of the ingredient following storage for up to 12 months under defined, accelerated storage conditions. 6'-SL was analyzed by HPAEC, and water content was analyzed by Karl Fischer titration at each time point.

The 6'-SL ingredient was stable throughout the 12-month storage period (for both Batches Nos. CPN5315 1000317 FD and CPN5315 1000617 FD) with no measurable loss of 6'-SL, other carbohydrates or change in

impurities content. As with the real-time stability testing, no appreciable changes in degradation of the ingredient or alterations in impurity profiles were observed. Based on the results of the accelerated stability study and taking the Arrhenius equation into account (Peleg *et al.*, 2012), the stability of the ingredient can be calculated to be at least 5 years when protected from light and stored at room temperature and ambient humidity.

Table 2.4.1.2-1 Results of 2-Year Accelerated Stability Study on 6'-SL (40°C, 75% Relative Humidity, RH) for 2 Representative Batches A) CPN5315 1000317 FD and B) CPN5315 1000617 FD

A) Manufacturing Batch Number CPN5315 1000317 FD

Parameter	Sample Time (Months)						
	0	1	2	3	6	9	12
Physical Properties							
Appearance	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated material, crystalline in appearance	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated material, crystalline in appearance
Color	White	White	White	White	White	White	White
Purity							
Assay (water free) HiMS [%]	101.3	101.2	99.2	96.8	100.0	99.6	97.4
Assay (water free) – 6'-SL [%]	97.8	97.8	95.8	93.5	96.7	96.3	93.9
Lactose [%]	2.80	2.86	2.83	2.75	2.73	2.82	2.86
Sialic acid [%]	0.59	0.48	0.49	0.48	0.50	0.49	0.53
6`-Sialyl-lactulose [%]	0.62	0.91	0.90	1.00	1.13	1.26	1.44
Largest unspecified impurity [%]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unspecified impurities [%]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Water content [%]	1.72	2.10	1.71	2.15	1.60	1.52	1.56
Microbiological Quality							
Aerobic mesophilic total plate count [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10	Not tested	< 10
Enterobacteriaceae	Not tested	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Salmonella spp.	Not tested	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Not tested	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Listeria monocytogenes	Not tested	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Bacillus cereus [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10	Not tested	< 10
Yeasts [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10	Not tested	< 10
Molds [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10	Not tested	< 10

6'-SL = 6'-sialyllactose sodium salt; CFU = colony forming units; HiMS = Human-identical Milk Saccharides = Sum of 6'-SL, lactose and sialic acid.

Table 2.4.1.2-1 Results of 2-Year Accelerated Stability Study on 6'-SL (40°C, 75% Relative Humidity, RH) for 2 Representative Batches A) CPN5315 1000317 FD and B) CPN5315 1000617 FD

B) Manufacturing Batch Number CPN5315 1000617 FD

Parameter	Sample Time (Months)						
	0	1	2	3	6	9	12
Physical Properties							
Appearance	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated material, crystalline in appearance	Uniformly sized, non-agglutinated powder	Uniformly sized, non-agglutinated material, crystalline in appearance
Color	White	White	White	White	White	White	White
Purity							
Assay (water free) HiMS [%]	97.8	98.6	98.4	97.0	99.7	98.0	99.4
Assay (water free) – 6'-SL [%]	94.4	95.2	95.1	93.7	96.4	94.7	96.0
Lactose [%]	2.49	2.55	2.51	2.51	2.46	2.44	2.58
Sialic acid [%]	0.89	0.76	0.76	0.77	0.78	0.74	0.77
6`-Sialyl-lactulose [%]	0.59	0.89	0.91	1.01	1.20	1.18	1.32
Largest unspecified impurity [%]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unspecified impurities [%]	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Water content [%]	1.50	1.99	1.44	1.73	1.70	1.53	1.55
Microbiological Quality							
Aerobic mesophilic total plate count [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10	Not tested	< 10
Enterobacteriaceae	Not tested	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Salmonella spp.	Not tested	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Not tested	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Listeria monocytogenes	Not tested	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Bacillus cereus [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10	Not tested	< 10
Yeasts [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10	Not tested	< 10
Molds [CFU/g]	Not tested	Not tested	Not tested	Not tested	< 10	Not tested	< 10

6'-SL = 6'-sialyllactose sodium salt; CFU = colony-forming units; HiMS = human-identical milk saccharides = Sum of 6'-SL, lactose and sialic acid.

2.4.1.3 Stress/Forced Stability

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

6'-SL Stability in Solid State

Forced, thermal stability tests of the bulk 6'-SL ingredient in powdered solid state were performed at 80°C for 28 days of storage at 2 different humidity conditions (ambient and high humidity).

The results of this study showed negligible concurrent increase of lactose and sialic acid during the storage period. Slight isomerization of 6'-SL to 6'-sialyl-lactulose was also observed, which was more pronounced in an increased humidity condition. The full study report can be provided upon request.

6'-SL Stability in Aqueous Solution

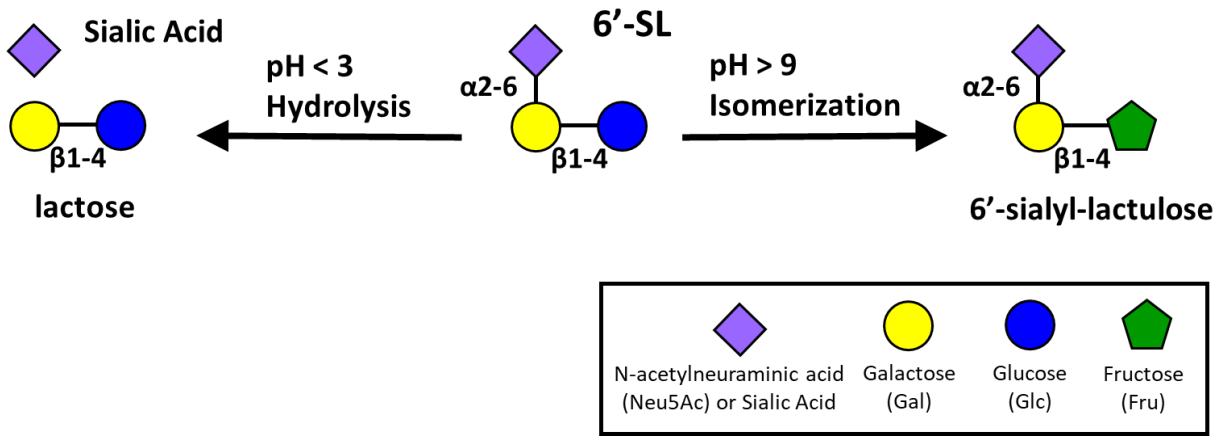
Forced stability tests of the bulk 6'-SL ingredient in aqueous solutions were conducted in exposure to the following stress conditions:

- Wide pH range (from 3.0 to 9.0) at 35°C over a period of 28 days.
- Acid (0.1 N HCl) and base (0.01 N NaOH) at 35°C over a period of 24 hours.

The results of this study showed the presence of 2 potential pH-dependent chemical pathways in the aqueous solutions of 6'-SL ingredient, namely hydrolysis at pH < 3.0 and isomerization at pH > 9.0 (Figure 2.4.1.3-1).

Based on the results of the study, 6'-SL in aqueous solution is stable at neutral pH (6.7 and 6.9) at 35°C for 1 month. In slightly acidic conditions (pH=5.0) at 35°C for 1 month, there was only minor (3%) hydrolysis of 6'-SL to sialic acid and lactose. However, 6'-SL almost completely hydrolyzed to sialic acid and lactose when exposed to acidic conditions (at pH 3.0, 35°C for 1 month or at 0.1 N HCl, 35°C for 24 hours). Under basic conditions (at pH 9.0, 35°C for 1 month or at 0.01 N NaOH 35°C for 24 hours), significant (10 to 30%) isomerization of 6'-SL to 6'-sialyl-lactulose was observed.

Figure 2.4.1.3-1 Degradation Pathways of 6'-Sialyllactose Sodium Salt (6'-SL) in Aqueous Solutions



2.4.2 Stability Under the Intended Conditions of Use

2.4.2.1 Stability in Powdered Infant Formula

The stability of the 6'-SL ingredient in powdered infant formula has been investigated using a HPLC method with fluorescent detection following long-term storage at various temperatures of 4, 20, 30 and 37°C.

The infant formula powder is a whey-based commercially available starter formula that is supplemented with 6'-SL and formulated together with other HiMOs to mimic their intended conditions of use in infant formula. The infant formula also contained long chain polyunsaturated fatty acids (LC-PUFA), vitamins, and minerals are present in the formula at concentrations intended for full nutritional support of infants from birth to 6 months of age. The interim results available to 12 months are presented below in Table 2.4.2.1-1. The stability of 6'-SL in commercially representative infant formula can be confirmed as the results demonstrate similarity between expected and observed values of the selected HMO (6'-SL) following 12 months storage under the tested conditions.

Table 2.4.2.1-1 Results of Stability of 6'-SL in a Commercially Representative Infant Formula Following Storage for up to 12 Months at Various Temperatures

			6'-SL
	Targeted concentration of HiMO per 100 g of IF		0.37
Sample time (months) and temperature (°C)	**T0**		0.42
	3 months	**4°C**	0.38
		20°C	0.39
		30°C	0.40
		37°C	0.40
	6 months	**4°C**	0.43
		20°C	0.37
		30°C	0.38
		37°C	0.38
	9 months	**4°C**	0.49
		20°C	0.49
		30°C	0.40
		37°C	0.45
	12 months	**4°C**	0.38
		20°C	0.37
		30°C	0.38
		37°C	0.36

6'-SL = 6'-sialyllactose sodium salt; IF = infant formula.

2.4.2.2 Stability in Other Food Matrices

The stability of a number of HiMS in other food matrices such as yogurts, ready-to-drink flavored milk, and citrus fruit beverages, has been evaluated previously and is presented in GRAS notifications for 2'-FL (GRN 546, 650, 735; U.S. FDA, 2014, 2015a, 2018d), LNnT (GRN 547, 659; U.S. FDA, 2015b, 2016c), and sialic acid (GRN 602; U.S. FDA, 2016a). A 3'-SL ingredient produced by GeneChem Inc. has GRAS status for use as an ingredient in non-exempt term infant formula and in dairy product analogs, infant and toddler foods, milk (whole and skim), milk products, grain products, beverages and beverages bases, and sugar substitutes at levels ranging from 24 to 3000 mg/RACC (GRN 766). Studies demonstrating the stability of 3'-SL in infant formula, milk and yogurt formulations have been previously reported by GeneChem Inc. and can be found in Section 2.C.5.2 of GRN 766. These studies demonstrate that 3'-SL, a structural isomer of 6'-SL, is expected to be stable in most food matrices.

Based on the accelerated stability information on 6'-SL and available stability information reported previously for 3'-SL, a structural isomer of 6'-SL and for related chemical structures that have been previously evaluated under the GRAS procedure, the conclusions on the stability of these HiMOs in food matrices can be extended to support the general stability of 6'-SL under the intended conditions of use.

Part 3. § 170.235 Dietary Exposure

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

3.1.1 6'-SL

To the best of our knowledge, the 6'-SL product described herein has not been determined to be GRAS before.

3.1.2 History of Consumption in Breast Milk

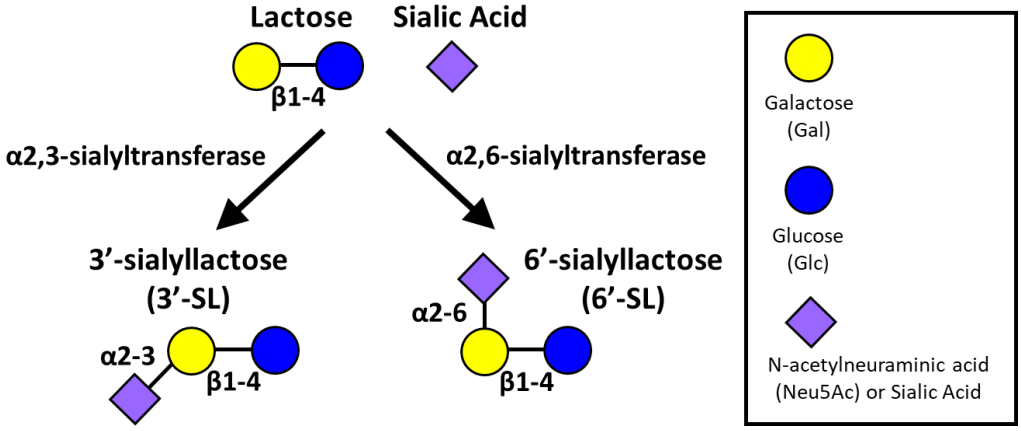
3.1.2.1 Human Biology Background Relevant to 6'-SL

6´-SL is an important component of the acidic (*i.e*., sialylated) 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) and since they occur in human milk at much higher concentrations than in any other mammalian milk (Urashima *et al.*, 2001). More than 160 members of this family have been fully described on a structural basis (Chen, 2015; Urashima *et al.*, 2018), 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 higher variations are observed on an individual level and in dependency of the lactation period and the genotype of the mother. In contrast, bovine milk contains approximately 20 times lower concentrations of a far less complex oligosaccharide mixture (Tao *et al.*, 2009; Aldredge *et al*., 2013; Urashima *et al*., 2013). The respective composition of each mammalian milk oligosaccharide fraction allows interesting insights into evolutionary aspects of lactation (Urashima *et al*., 2012).

6´-SL belongs to the acidic sub-fraction of HMOs, which are oligosaccharides that contain the acidic carbohydrate sialic acid[10] and are therefore also called "sialylated HMOs". They are reported to constitute, on average, around 15% of the total HMO fraction (Bode *et al*., 2012). The sialylated 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 sialyltransferases. Dependent on the enzyme specificity (for example α2,3-or α2,6-sialyltransferase) lactose can be sialylated (*i.e*., "decorated" with sialic acid) at the 3 or 6 position of the galactose unit (Figure 3.1.3.1-1), forming 3'-SL or 6'-SL , respectively (α2,3-/α2,6-sialyllactose), the 2 predominant forms of sialyllactose found in milk (Martín-Sosa *et al.,* 2003; Sprenger *et al.,* 2017).

[10] Sialic acid is the more common (but colloquial) synonym for *N*-acetylneuraminic acid (abbreviated either as NANA or Neu5Ac). NANA has GRAS status for use as an ingredient in term infant formula at a maximum use level of 50 mg/L (GRN 602 – U.S. FDA, 2016a).

Figure 3.1.3.1-1 Sialylation of Lactose by Specific Sialyltransferases



Sialyllactoses are linked to many important biological functions since sialylation of glycoconjugates on cell surfaces is a central biological principle observed in many lineages of life, most importantly in the superphylum of deuterostomes, and particularly in the clade of chordata, which includes mammals (Bishop and Gagneux, 2007). In consequence, mammalian cells are covered by a dense layer of sialylated glycoconjugates and receptors that are able to recognize them (*e.g.*, siglecs and selectins) (Ley, 2003; Macauley *et al.*, 2014). Therefore, sialylated glycoconjugates play central biological roles in cell-cell communication, cell development, cell clearance, immunity, infection and cognitive development (Schauer, 2004; Varki and Gagneux, 2012; Wang, 2012).

The structural identity of the freely occurring HMOs 3'- and 6'-sialyllactose to the same structural carbohydrate structures on cell surfaces has the consequence that 3'-SL and 6'-SL also act as ligands for receptor binding and often exhibit related functions as the linked glycoconjugates that they mimic.

For instance, many pathogens (viral, bacterial and protozoan) and their toxins bind specifically to 3'- or 6'-sialylo-glycoconjugates and use the selective binding and hydrolysis of sialic acid as a mechanism to enter and escape a cell after replication (ten Bruggencate *et al.*, 2014; Matrosovich *et al.*, 2015). Probably the most prominent examples are the respective influenza proteins: *haemagglutinin* (H) that is responsible for sialic acid binding and *neuraminidase* (N) for hydrolysis of sialic acid (these are used in the HxNy strain nomenclature well-known from influenza vaccines). 3'-SL and 6'-SL have been reported to bind to the same pathogens and to show promise in preventive treatment as anti-adhesives (ten Bruggencate *et al.*, 2014).

Furthermore, human sialic acid biology is remarkable in some respects:

- The expression of α2,6-linked sialic acids has been upregulated in humans in comparison to other mammals, including our closest living relatives, the great apes (Gagneux *et al.*, 2003). Consequently, human epithelial airway cells contain more α2,6-linked than α2,3-linked sialic acids with consequences for susceptibility to the human influenza virus (Cohen *et al.*, 2013).

- Human milk contains significantly more 6'-sialyllactose than 3'-sialyllactose, in contrast to other mammalian milks, where 3'-SL is the predominant sialyllactose (Urashima *et al.*, 2001; ten Bruggencate *et al.*, 2014; Thurl *et al.*, 2017).

- In recent evolution humans have lost the ability to metabolically convert Neu5Ac to a hydroxylated derivative called N-glycolylneuraminic acid (Neu5Gc) (Chou *et al.*, 1998; Irie *et al.*, 1998). In

consequence, sialyllactose of human milk contains the sialic acid as highly pure Neu5Ac form, whereas other mammalian milks typically contain some levels of Neu5Gc (Urashima *et al.*, 2001; Röhrig *et al.*, 2017).

- Dietary intake of Neu5Gc has recently been recognized as a risk factor for a number of diseases including inflammation and cancer (Samraj *et al.*, 2015).

- The highest concentrations of sialic acid in the human body are found in the brain and in breastmilk (Röhrig *et al.*, 2017), and levels of sialic acid in breastmilk vs infant formula have been corelated to better cognitive outcomes in breastfed infants (Wang, 2009).

In summary, we conclude that the HiMO 6'-SL constitutes a highly characteristic molecule for human nutrition in general, and infant nutrition in particular.

3.1.2.2 Quantity of 6'-SL in Breast Milk

The concentration of 6'-SL in human milk has been measured and reported to date in at least 18 independent publications (references included in Table 3.1.3.2-1). The data demonstrate that 6'-SL is the major and, on average, the most abundant sialylated HMO of human milk and among the most abundant HMOs in general. Table 3.1.3.2-1 summarizes the levels of 6'-SL that have been reported in breast milk across these various studies.

The **average** 6'-SL levels in human milk gradually decline throughout the lactation period, starting at concentrations of 0.54 g/L in colostrum, followed by 0.45 g/L and 0.39 g/L in transitional and mature milks, respectively, and then further decline in mature milk from a lactation stage later than 2 months (0.12 g/L). However, the reported ranges of 6'-SL in human milks widely vary from 0.13 and 1.31 g per liter of milk.

Table 3.1.3.2-1 The 6'-SL Concentration in Human Milk after Full-Term Birth

Lactation Time	Key Findings	References
Pooled Milk		
Days 1 to 4 ("colostrum")	**Reported Range: 0.3 to 1.3 g/L** Average: 0.54 g/L	Coppa *et al*. (1999); Kunz *et al.* (2000); Martín-Sosa *et al.* (2003); Bao *et al.* (2007); Asakuma *et al.* (2007); Thurl *et al.* (2010); Spevacek *et al.* (2015); Kunz *et al.* (2017); Nijman *et al.* (2018)
Days 5 to 14 ("transitional milk")	**Reported Range: 0.2 to 0.7 g/L** Average: 0.45 g/L	Coppa *et al*. (1999); Kunz *et al.* (2000); Martín-Sosa *et al.* (2003); Sakaguchi *et al*. (2014); Spevacek *et al.* (2015); Austin *et al.* (2016); Kunz *et al.* (2017)
Days 10 to 60 ("mature milk")	**Reported Range: 0.1 to 1.3 g/L** Average: 0.39 g/L	Kunz and Rudloff (1993); Thurl *et al.* (1996); Coppa *et al.* (1999); Martín-Sosa *et al.* (2003); Bao *et al.* (2007); Thurl *et al.* (2010); Leo *et al.* (2010); Hong *et al.* (2014); Spevacek *et al.* (2015); Austin *et al.* (2016); Kunz *et al.* (2017); Sprenger *et al.* (2017); McGuire *et al.* (2017); Nijman *et al.* (2018)
After 2 months ("mature milk")	**Reported Range: 0.05 to 0.2 g/L** Average: 0.12 g/L	Coppa *et al*. (1999); Smilowitz *et al.* (2013); Sakaguchi *et al*. (2014); Austin *et al.* (2016); Sprenger *et al.* (2017)

6'-SL = 6'-sialyllactose sodium salt.

The reported concentrations of sialylated HMOs in human milk are similar among mothers with secretor or non-secretor phenotype (Xu *et al.*, 2017; Elwakiel *et al.*, 2018). A recently published systematic review of the concentrations of oligosaccharides in human milk (Thurl *et al.*, 2017) reported the content of 6'-SL in secretor milk at 0.64 g/L (with 95% confidence limits of 0.38 to 0.91) and at 0.35 g/L (with 95% confidence limits of 0.29 to 0.42) in pooled milk. However, even though this meta-analysis showed a clear numerical

difference in the content of selected individual sialylated HMOs in milks from secretors and non-secretors, it has been concluded that there was no general tendency observed toward the levels of the sialylated HMO sub-fraction in the milks of mothers of varied phenotypes.

Only 1 study has investigated the regional (ethnic) dependency of the 6'-SL concentrations of milk under comparable conditions (McGuire *et al.,* 2017). In this study, 6'-SL appeared to be highly influential to the variability of HMO profiles in the groups from rural Ethiopia, USA California (Hispanic), USA Washington, and Peru, but not in the other studied cohorts, including urban Ethiopia, rural and urban Gambia, Ghana, Kenya, Spain, and Sweden. In terms of the concentrations of 6'-SL significantly highest and lowest values of 0.54 and 0.13 g/L, were reported for Ghana and Sweden, respectively. However, this data is based on a single study and time of sampling was not strictly controlled between different regions (the inclusion criterium was that samples were collected between 2 weeks and 5 months postpartum, and a bias toward earlier or later milk samples within a cohort would have a large influence on concentrations of 6'-SL within a population); therefore, it is possible that the reported low and high extremes may be sample-biased, rather than real differences (Newburg, 2017).

When taking the whole data from all studies listed in Table 3.1.3.2-1 into account, the average 6'-SL concentrations in various regions would vary from 0.25 ± 0.18 g/L in Asian countries, through 0.34 ± 0.17 g/L in U.S., 0.35 ± 0.12 g/L in Africa to 0.49 ±0.38 g/L in Europe and 0.63 ± 0.41 g/L in Latin America (Peru). The different numerical values overlap when considering the standard deviations, hence, the most proportionate conclusion is that there is a wide variation between individual mothers that covers ranges up to more than 1.3 g/L of 6'-SL.

Using the range of ***average*** levels of 6'-SL reported in breast milk over different times of lactation, of 0.12 to 0.54 g/L (see Table 3.1.3.2-1), combined with the estimated high consumption formula intake value for young infants of 260 mL/kg body weight/day (EFSA, 2017), the level of 6'-SL from breastfeeding can be estimated at between **31 to 140 mg/kg body weight/day**.

Using the upper reported range of 6'-SL in breast milk over different times of lactation, of 1.3 g/L (see Table 3.1.3.2-1), combined with the estimated high consumption formula intake value for young infants of 260 mL/kg body weight/day (EFSA, 2017), the level of 6'-SL from breastfeeding can be estimated to be **330 mg/kg body weight/day**.

3.1.3 History of Commercial Use of 6'-SL and Other HiMOs

6'-SL manufactured by Glycom has no history of commercial use; however, 3'-SL, a structural isomer of 6'-SL, produced by GeneChem Inc. using enzymatic synthesis methods has GRAS status for use as an ingredient in non-exempt term infant formula at a maximum level of 238 mg/L as consumed. This ingredient also is GRAS for use as an ingredient in dairy product analogs, infant and toddler foods, milk (whole and skim), milk products, grain products, beverages and beverages bases, and sugar substitutes at levels ranging from 24 to 3000 mg/RACC. GeneChem's conclusions on the GRAS status of 3'-SL was submitted to the offices of the U.S. FDA under the voluntary GRAS notification procedure and was filed by the agency without objection under GRN 766. At this time, Glycom is not aware of any infant formula preparations containing 6'-SL that have been introduced to the U.S. marketplace. 6'-SL manufactured by Glycom will be used in a substitutional manner to other 6'-SL ingredients that have GRAS status.

3.2 Estimated Intake of 6'-SL

3.2.1 Methods

An assessment of the anticipated intake of 6'-SL as an ingredient under the intended conditions of use (see Table 1.3-1) 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 6'-SL for each of the following population groups:

- Infants, ages 0 to less than 1;
- 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 6'-SL by the U.S. population[11]. Estimates for the daily intake of 6'-SL 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 6'-SL averaged over all individuals surveyed, regardless of whether they consumed food products in which 6'-SL is proposed for use, and therefore includes individuals with "zero" intakes (*i.e.*, those who reported no intake of food products containing 6'-SL during the 2 survey days). "Consumer-only" intake refers to the estimated intake of 6'-SL by those individuals who reported consuming food products in which the use of 6'-SL is currently under consideration. Individuals were considered "consumers" if they reported consumption of 1 or more food products in which 6'-SL is proposed for use on either Day 1 or Day 2 of the survey.

[11] Statistical analysis and data management were conducted in DaDiet Software (Dazult Ltd., 2018). DaDiet Software is a web-based software tool that allows accurate estimate of exposure to nutrients and to 6'-sialyllactose added to foods, including contaminants, food additives and novel ingredients. The main input components are concentration (use-level) data and food consumption data. Data sets are combined in the software to provide accurate and efficient exposure assessments.

The estimates for the intake of 6'-SL 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 assessment are presented in Section 3.2.2.

3.2.2 Intake Estimates for 6'-SL

A summary of the estimated daily intake of 6'-SL 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 80.1% of the population groups consisted of consumers of food products in which 6'-SL is currently proposed for use (Table 3.2.2-1). Infants aged 7 to <12 months had the greatest proportion of consumers at 99.9%. 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 6'-SL were determined to be 0.41 and 0.89 g/person/day, respectively. Of the individual population groups, infants aged 7 to <12 months were determined to have the greatest mean and 90th percentile consumer-only intakes of 6'-SL on an absolute basis, at 0.88 and 1.64 g/person/day, respectively. The elderly had the lowest mean consumer-only intake of 0.33 g/person/day, while female teenagers had the lowest 90th percentile consumer-only intakes of 0.69 g/person/day (Table 3.2.2-1).

Table 3.2.2-1 Summary of the Estimated Daily Intake of 6'-SL[a] from Proposed Food-Uses in the U.S. by Population Group (2013-2014 NHANES Data)

Population Group	Age Group (Years Unless Otherwise Specified)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 months	0.49	1.02	80.1	165	0.61	1.10
Infants	7 to <12 months	0.87	1.64	99.9	127	0.88	1.64
Toddlers	1 to 3	0.44	0.93	98.5	465	0.45	0.94
Children	4 to 10	0.37	0.73	99.0	986	0.37	0.73
Female Teenagers	11 to 18	0.33	0.68	94.5	572	0.35	0.69
Male Teenagers	11 to 18	0.46	0.81	98.2	570	0.46	0.83
Female Adults of Childbearing Age	19 to 40	0.36	0.78	92.9	826	0.39	0.78
Female Adults	19 to 64	0.37	0.84	92.9	1,764	0.40	0.86
Male Adults	19 to 64	0.43	0.99	92.7	1,522	0.47	1.01
Elderly	65 and up	0.31	0.74	92.2	917	0.33	0.76
Total Population	All ages	0.39	0.86	93.8	7,088	0.41	0.89

6'-SL = 6'-sialyllactose sodium salt; 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 total population (all ages) mean and 90th percentile consumer-only intakes of 6'-SL were determined to be 8.6 and 16.8 mg/kg body weight/day, respectively. Among the individual population groups, infants aged 7 to <12 months were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, of 98.7 and 176.0 mg/kg body weight/day,

respectively. The elderly had the lowest mean and 90th percentile consumer-only intakes of 4.4 and 10.3 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 6'-SL[a] from Proposed Food-Uses in the U.S. by Population Group (2013-2014 NHANES Data)

Population Group	Age Group (Years Unless Otherwise Specified)	*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 months	70.5	143.0	80.1	165	88.0	151.0
Infants	7 to <12 months	98.5	176.0	99.9	127	98.7	176.0
Toddlers	1 to 3	34.6	70.5	98.5	460	35.1	70.5
Children	4 to 10	13.7	28.0	98.9	980	13.8	28.2
Female Teenagers	11 to 18	5.8	13.0	94.6	568	6.2	13.2
Male Teenagers	11 to 18	7.2	14.0	98.2	569	7.3	14
Female Adults of Childbearing Age	19 to 40	5.0	11.0	92.9	819	5.4	11.4
Female Adults	19 to 64	4.9	11.3	92.9	1,752	5.3	11.6
Male Adults	19 to 64	5.1	11.9	92.7	1,518	5.5	12.5
Elderly	65 and up	4.0	10.2	92.1	906	4.4	10.3
Total Population	All ages	8.1	16.0	93.8	7,045	8.6	16.8

6'-SL = 6'-sialyllactose sodium salt; 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.

3.2.3 Summary and Conclusions

Consumption data and information pertaining to the individual proposed food-uses of 6'-SL were used to estimate the *per capita* and consumer-only intakes of 6'-SL (ingredient on a wet weight basis) for specific demographic groups and for the total U.S. population. There were several assumptions included in the assessment which render exposure estimates that may be considered suitably conservative. For example, it has been assumed in the exposure assessment that all food products within a food category contain 6'-SL 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 6'-SL will have 100% market penetration in all identified food categories.

In summary, on consumer-only basis, the resulting mean and 90th percentile intakes of 6'-SL by the total U.S. population from all proposed food-uses, were estimated to be 0.41 g/person/day (8.6 mg/kg body weight/day) and 0.89 g/person/day (16.8 mg/kg body weight/day), respectively. Among the individual population groups, the highest mean and 90th percentile consumer-only intakes of 6'-SL were determined to be 0.88 g/person/day (98.7 mg/kg body weight/day) and 1.64 g/person/day (176.0 mg/kg body weight/day), respectively, as identified among infants aged 7 to <12 months. The elderly had the lowest mean consumer-only intakes of 0.33 g/person/day (4.4 mg/kg body weight/day), while female teenagers had the lowest 90th percentile consumer-only intakes of 0.69 g/person/day (13.2 mg/kg body weight/day). When intakes were expressed on a body weight basis, infants aged 7 to <12 months had the highest mean and 90th percentile consumer-only intakes of 98.7 and 176.0 mg/kg body weight/day, respectively.

Part 4. § 170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with 6'-SL.

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

Not applicable.

Part 6. § 170.250 Narrative and Safety Information

6.1 Introduction

Glycom has conducted a scientific procedures GRAS evaluation of 6'-SL for use as an ingredient in infant formula and specified conventional food applications targeted to the general public. 6'-SL as manufactured by Glycom has been demonstrated to be identical in structure to its natural counterpart secreted into human milk and therefore can be referred to as a HiMO. The ingredient will be added to infant formula at levels that will result in concentrations that are within the reported mean concentrations that have been measured in pooled breast milk samples obtained from lactating women across all lactational stages and therefore the safety of adding 6'-SL to infant formula is supported by pivotal information establishing its history of safe consumption by breast-feeding infants. As infants are a sensitive population group, the safety of dietary ingestion of HiMOs from breast milk consumption also can be extended to adults consuming HiMOs at comparable ingestion levels in conventional food products.

Since 6'-SL is intended for addition to infant formula at levels that are within the ranges that have been reported in human breast milk samples, toxicological evaluations in rats, or tolerance studies in neonatal piglets, were not necessary to establish an appropriate margin of safety for exposures to 6'-SL from the intended food uses. Notwithstanding these conclusions, product specific toxicological data that has been obtained on 6'-SL manufactured by Glycom provides corroborating information on the safety of the company's production processes including the safety of the production strain. Phipps *et al.* (2019a) reported findings from a toxicological battery that included an adapted subchronic (90-day) oral toxicity study with neonatal rats, a bacterial reverse mutation assay, and an *in vitro* mammalian cell micronucleus test in human lymphocytes conducted using 6'-SL manufactured by Glycom. A no-observed-adverse-effect level (NOAEL) of 5,000 mg/kg body weight/day, the highest dose tested, was reported by the authors and the ingredient was without evidence of genotoxicity/mutagenicity. These studies are discussed in greater detail in Section 6.4. Studies evaluating the acute, subchronic and genotoxicity a 6'-SL ingredient manufactured by GeneChem were without evidence of toxicity (Gurung *et al*., 2018a). A mixture of 6'-SL and 3'-SL manufactured by Arla also was demonstrated to be well tolerated in neonatal piglets (Monaco *et al*., 2018).

Toxicity studies of 3'-SL, an isomer of 6'-SL, were also reviewed by Glycom. 3'-SL and 6'-SL are expected to share similar biological functionalities, and therefore, the toxicological data obtained for 3'-SL ingredients are considered relevant to the hazard characterization of 6'-SL. A NOAEL of 5,000 mg/kg body weight/day, the highest dose tested, can be derived for 3'-SL. These studies are discussed in more detail in Section 6.4.2. Glycom also reviewed published studies evaluating the safety a 3'-SL ingredient manufactured by GeneChem and included a toxicological battery in weanling rats, beagle dogs (Kim *et al*., 2018), and a tolerance study in piglets (Monaco *et al*., 2019) (see Section 6.4.4.2.4). A NOAEL of 2,000 mg/kg body weight per day the highest dose tested was derived for 3'-SL from the subchronic rat study and consumption of 3'-SL by piglets at doses up to 253 mg/kg body weight/day was well tolerated over a 4-week treatment interval. These studies on 3'-SL corroborate the GRAS status of 6'-SL.

No human studies have been conducted using 6'-SL; however, one relevant clinical study assessing the safety and gastrointestinal tolerance of 3'-SL has been reported (Section 6.5.2). Male and female *Helicobacter pylori* positive (but otherwise healthy) subjects were provided with 12 g of 3'-SL or a placebo power daily in 3 servings (4 g immediately after breakfast, lunch, and dinner) for 4 weeks (Gurung *et al*., 2018b). There were no statistically significant differences in adverse events, gastrointestinal (GI) symptoms, physical examinations, or clinical pathology values. Thus, this clinical study directly demonstrates the safety and tolerability of 3'-SL, and indirectly the safety and tolerability of 6'-SL.

Glycom noted that 6'-SL may be used by infant formula manufacturers in various combinations with other HiMO ingredients that have GRAS status for use in infant formula. As all of Glycom's HiMOs will be used in infant and toddler formula at concentrations that are consistent with levels in human milk there is no safety concern for excessive exposure by infants to HiMOs from infant formula manufacturers using these ingredients in various combinations. There is potential for additive consumption of HiMOs from the introduction of other HiMO containing foods to the infants' diet as they age; however, HiMOs are not absorbed and are fermented in the gastrointestinal tract to innocuous metabolites. Moreover, there is a very large natural variation in the HiMO concentrations that have been reported in the literature supporting that large variations in levels and ratios of HiMOs relative to one another are well tolerated by infants. The total concentration of HMOs in human breast milk has been reported to be as high as 25 g per liter of human milk (Gabrielli *et al.,* 2011), a value that demonstrates that these dietary substances are well tolerated by infants and young children at high levels.

Finally, Glycom evaluated the allergenicity risk of 6'-SL (Section 6.6). As a purified ingredient, 6'-SL manufactured by Glycom is absent of detectable levels of protein based on the results of the modified Bradford method at a detection limit of 17 ppm. The amino acid sequences of all introduced proteins were evaluated using Allergen Online tool (version 18B) hosted by the University of Nebraska's Food Allergen Research and Resource Program (FARRP, 2018). No positive alignments between any of the recombinant proteins and known/putative allergen sequences within the database were identified. 6'-SL manufactured by Glycom was concluded to be of low allergenic risk.

6.2 Literature Search

Glycom considered the totality of publicly available data and information relevant to the safety of 6'-SL. A comprehensive and detailed search of the published scientific literature was conducted through July 2019 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 6'-SL have considered all publicly available sources of information including favorable and potentially

unfavorable information. Based on Glycom's updated search of the literature, the company is not aware of published studies to suggest the 6'-SL is unsafe for use as a food ingredient.

6.3 Absorption, Distribution, Metabolism, and Excretion

6'-SL manufactured by Glycom has been determined analytically to be structurally identical to its naturally occurring counterpart in human milk. The absorption, distribution, and metabolism of HMOs have been the subject of extensive investigation (Brand-Miller *et al.*, 1995, 1998; Engfer *et al.*, 2000; Gnoth *et al*., 2000; Chaturvedi *et al*., 2001; Rudolff and Kunz, 2012) and it can be concluded that HMOs, including 6'-SL, do not undergo any significant digestion in the upper gastrointestinal tract. Very small quantities of ingested HMOs have been reported to be absorbed intact (approximately 1 to 2% of the total amount of HMO ingested) and are excreted unchanged in urine. The absorption of the 6'-SL added to infant formula would be limited and quantities absorbed would be no different to that occurring in infants consuming human breast milk. For a more comprehensive discussion of the absorption, distribution, metabolism, and excretion (ADME) profile of other HiMOs, the reader is directed to GRN 546, 547, 650, and 659 (U.S. FDA, 2015a,b, 2016b,c).

6.4 Toxicology Studies

The risk assessment approach for 6'-SL follows the same procedures used to support the safety of other HiMOs that have been previously determined to be GRAS (GRN 547, 659; U.S. FDA, 2015b, 2016b). Pivotal data and information supporting the safety of Glycom's HiMO ingredients are based on qualitative data establishing that HiMOs manufactured by Glycom are chemically identical to those present within human milk and the fact that the intended uses of 6'-SL in infant formula are equivalent to mean levels that have been quantitated for human milk samples across all lactational stages. Since all of Glycom's HiMOs are intended to be used alone or in combination with other HiMOs at levels that are individually or cumulatively within the range that has been reported in human milk samples, the risk assessment does not require derivation of a margin of safety for exposure to the ingredients from infant formula use relative to a NOAEL value from toxicological investigation. Toxicity studies of HiMOs manufactured by Glycom therefore largely corroborative in nature, demonstrating that non-HiMO constituents originating from the fermentation organism are not present at levels of toxicological concern. To date, 6 toxicity studies in neonatal rats have been conducted using production strains derived from Glycom's *E. coli* K-12 DH1 MDO lineage (Coulet *et al.,* 2013, 2014; Phipps *et al.,* 2018a,b, 2019a,b). Clear evidence of test article related toxicity has not been reported in any of these studies up to the highest doses that have been tested. The results of toxicity studies conducted with 6'-SL and its isomer 3'-SL are reported below.

6.4.1 Studies Conducted with Glycom's 6'-SL

6.4.1.1 Repeat Dose Toxicity Studies

6.4.1.1.1 14-Day Toxicity Study in the Neonatal Rat

A 14-day repeat dose toxicity study was conducted in rats to evaluate the potential short-term toxicity of 6'-SL and to select dose levels for the subsequent 90-day study (Phipps *et al*., 2019a).

Groups of 8 male and 8 female neonatal rats were dosed starting on Post Natal Day (PND) 7 with 0 (water for irrigation), 4,000, or 5,000 mg/kg body weight/day of 6'-SL, by gavage at a dose volume of 10 mL/kg body weight, once daily for 14 days, until the day before necropsy. Doses of 6'-SL were corrected to account for "other carbohydrates" within the test article batch.

All animals were observed 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 deaths and no test item-related clinical signs. On the first day of dosing (Day 7 of age) 4 pups [1 control female and 3 animals (1 male and 2 females) receiving 4,000 mg/kg body weight/day], were noted to have slight dose reflux immediately after the dosing procedure. Also, on the first day of dosing, 1 male receiving 5,000 mg/kg body weight/day gasped for approximately 10 seconds immediately after the dosing procedure. These observations were considered to be incidental and unrelated to the test item, as all of these animals were observed as normal at the 'end of group' dose observation on that day and no further clinical observations were seen for the remainder of the study.

There were no test item-related differences in body weight between 6'-SL-treated groups and controls. Group mean body weights for females given 4,000 mg/kg body weight/day were 10% higher than those of controls at the end of the treatment period. However, this was primarily due to females given 4,000 mg/kg body weight/day being 7% heavier than controls on the first day of dosing. The overall body weight gain for females given 4,000 mg/kg body weight/day was also slightly higher (11%) than controls, but there was no evidence of a dose-response (females given 5,000 mg/kg body weight/day were only 6% heavier than controls at the end of the dosing period and had gained only 8% more weight overall). Therefore, these differences were considered to be unrelated to the test item. No test item-related macroscopic abnormalities were observed.

<u>In 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.</u>

6.4.1.1.2 90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of 6'-SL when administered by gavage to neonatal rats from PND 7 (Phipps *et al.,* 2019a). The study was conducted in compliance with the Organisation for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b), but was adapted by using neonatal animals (as 6'-SL is intended for use in infant formula) to consider the requirements of EFSA *Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age* (EFSA, 2017*), Guidance for industry: nonclinical safety evaluation of paediatric drug products* (U.S. FDA, 2006), *Guideline on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications* (EMEA, 2008), and the *Guideline on the Nonclinical Safety Study in Juvenile Animals for Paediatric Drugs* (MHLW, 2018).

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 1,000, 3,000, or 5,000 mg/kg body weight/day 6'-SL, 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 6'-SL group and identify any effects related to the general fiber-like characteristics of the reference material. Doses of 6'-SL and reference control were corrected to account for "other carbohydrates" within the test article batch. An additional 5 males and 5 females in each group were also dosed once daily for at least 90 days and then kept un-dosed for 4 weeks, to assess the reversibility of any observed effects.

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 6'-SL 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; water consumption was recorded 1 week before urine collection on each occasion.

In Weeks 11 and 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 surviving animals (at the end of the treatment and recovery periods) were subjected to a gross macroscopic necropsy, where selected organs (adrenal glands, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary gland, prostate, submandibular and sublingual salivary glands, seminal vesicles, spleen, testes, thymus, thyroid/parathyroid glands and uterus/cervix) were weighed and fixed. At the end of the treatment period, a full list of tissues [adrenal glands, aorta, brain, caecum, colon, duodenum, epididymides, eyes, femur, Harderian glands, head, heart, ileum, jejunum, kidneys, liver, lungs, mesenteric and left axillary lymph nodes, esophagus, ovaries, pancreas, pituitary gland, prostate, salivary glands, sciatic nerves, seminal vesicles, skeletal muscle, skin (with mammary glands), spinal cord, spleen, sternum, stomach, testes, thymus, thyroid glands (with parathyroids), trachea, urinary bladder, uterus (with cervix) and vagina] for early decedents and animals in the vehicle control and high-dose 6'-SL, were examined microscopically. The testes and epididymides were examined microscopically at the end of the treatment period (for all male groups) and at the end of the treatment-free period (for vehicle control, reference control and high-dose males).

There were no test item-related deaths, clinical signs, or ocular changes. One reference control male was euthanized on Day 88 of dosing after showing clinical signs including gasping and unresponsiveness. The only notable macroscopic findings were depressions on the kidneys, which correlated with a minimal severity infiltrate of mononuclear inflammatory cells in the renal cortex seen microscopically. There were no other notable microscopic findings and the cause of the animal's poor clinical condition could not be identified, but as it was an isolated instance it was considered unrelated to administration of the reference control. One male given 5,000 mg/kg body weight/day was found dead on Day 20 of dosing, with no notable macroscopic or microscopic findings reported. As it was an isolated instance it was considered unrelated to administration of 6'-SL.

No biologically relevant differences in body weight or food consumption between 6'-SL-treated groups and controls were observed.

6'-SL administration had no effect 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). Ulna length and growth were similar between 6'-SL-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 were observed. Morris maze performance was also unaffected by administration of 6'-SL, 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). Where statistically significant differences were observed, they were not associated with a dose-response (longer time of completion for balano preputial separation for males given 1,000 mg/kg body weight/day, shorter time of completion for vaginal opening for females given 3,000 mg/kg body weight/day, lower body weight at time of vaginal opening for all 6'-SL-treated female groups).

No test item-related differences in values for hematological parameters between 6'-SL-treated groups and vehicle controls were observed. At the end of the treatment period, prothrombin time was statistically significantly shorter for males given 3,000 or 5,000 mg/kg body weight/day and females given 5,000 mg/kg body weight/day, compared with vehicle controls. Most of the individual values for 6'-SL-treated males were within the historical control data (HCD) range (2 vehicle control male values were outside the HCD range, compared to only 1 of the individual male values at 5,000 mg/kg body weight/day), which indicates that values were generally within normal biological variation. For females, there was no dose-response relationship. The high-dose 6'-SL male and female mean values (20.4 and 19.2 seconds, respectively) were also both comparable with the respective values for reference control males and females (20.5 and 19.8 seconds, respectively). Other statistically significant differences were also clearly unrelated to the test item, as they were not associated with a dose-response [reduced hemoglobin for males in all 6'-SL groups and females given 5,000 mg/kg body weight/day; reduced hematocrit and red blood cells (RBC) for females given 5,000 mg/kg body weight/day; lengthened activated partial thromboplastin time (APTT) for females given 5,000 mg/kg body weight/day; reduced platelets for all female 6'-SL groups; increased eosinophils for females given 5,000 mg/kg body weight/day].

There were no test item-related effects on blood chemistry parameters. At the end of the treatment period, chloride was statistically significantly reduced for males and females receiving 3,000 or 5,000 mg/kg body weight/day. Where other statistically significant findings were observed, they were not associated with a dose-response [increased aspartate transaminase (AST) for all male 6'-SL groups; increased albumin/globulin (A/G) ratio for males given 3,000 or 5,000 mg/kg body weight/day; reduced total protein for all male and female 6'-SL groups; reduced bilirubin for males given 1,000 or 3,000 mg/kg body weight/day; reduced cholesterol for males and females given 5,000 mg/kg body weight/day; reduced albumin for all female 6'-SL groups] and/or were inconsistent between the sexes (reduced potassium for males given 5,000 mg/kg body weight/day and reduced sodium for females given 3,000 or 5,000 mg/kg body weight/day).

There were no test item-related differences in urinalysis parameters between 6'-SL-treated groups and controls. Total urinary protein was statistically significantly reduced for male and female animals receiving 5,000 mg/kg body weight/day, but for males there was no dose-response. Furthermore, all individual male values were within the HCD range and the same number of individual female vehicle control values (2) were outside the HCD range as in the 5,000 mg/kg body weight/day group, indicating that high-dose values reflected normal biological variation.

Organ weights were unaffected by 6'-SL administration. Where statistically significant differences were observed, the differences were either not associated with a dose-response (reduced body weight adjusted salivary glands for females receiving 3,000 or 5,000 mg/kg body weight/day) or were only seen in 1 sex (increased body weight adjusted liver weights for males receiving 3,000 or 5,000 mg/kg body weight/day; reduced body weight adjusted heart weights for females receiving 3,000 or 5,000 mg/kg body weight/day). As there was no effect on the pituitary-thyroid axis observed during the study, the samples collected for potential analysis of thyroid stimulating hormone (TSH), T3 and T4 were not analyzed; this is in accordance

with OECD Test Guideline 407 (OECD, 2008), which the EFSA *Guidance for submission for food additive evaluations* refers to regarding modification of OECD Test Guideline 408 (OECD, 1998b) studies, to include assessment of some additional parameters that place more emphasis on endocrine-related endpoints (EFSA, 2012).

There were no biologically relevant macroscopic or microscopic findings at 1,000 or 3,000 mg/kg body weight/day. Four males given 5,000 mg/kg body weight/day had unilateral tubular atrophy in the testis and absence of sperm in the epididymis on the same side. These findings were considered unrelated to treatment and are discussed in detail by Phipps *et al*., (2019b). In brief it was concluded that the unilateral nature of the findings combined with the fact that some degree of mild to moderate histopathological degeneration would have been observed in at least some of the animals in the lower dose groups given the severity of the findings in the high dose animals support a conclusion that the findings were not test article related. Chemical induced testicular toxicity resulting in absence of sperm in the epididymides also would be expected to be a chronic progressive effect that would manifest to some severity level in the lower dose-groups. In addition, the severity of the findings and corresponding absence of similar effects at any severity level in any of the recovery animals further suggests that the effects were a background genetic aberration and unrelated to 6'-SL treatment. Finally, the absence of similar findings in another subchronic toxicity study of 6'-SL reported by Gurung *et al*., (2018a) and of studies conducted with its constitutional isomer 3'-SL also corroborate the authors conclusions.

In absence of any test item-related adverse effects at lower doses, the NOAEL was concluded to be 5,000 mg/kg body weight/day 6'-SL, the highest dose tested.

6.4.1.2 Genotoxicity Studies

6.4.1.2.1 Bacterial Reverse Mutation Test

The potential mutagenicity of 6'-SL was evaluated 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[12] B13/14, U.S. EPA Health Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998) and FDA Redbook IV.C.1.a. (U.S. FDA, 2000a) (Phipps *et al.,* 2019a).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* (*E. coli*) strain WP2 uvrA (pKM101), which were treated with 6'-SL at concentrations of up to 5,000 µg/plate (the OECD 471 guideline maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix).

Water (purified by reverse osmosis) served as the vehicle for 6'-SL 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 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.

[12] EC (2008). 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.

There was no evidence of mutagenicity following exposure to 6'-SL in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in revertant colony counts (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that 6'-SL is non-mutagenic at concentrations up to 5,000 µg/plate (the OECD 471 guideline maximum recommended concentration).

6.4.1.2.2 *In Vitro* Mammalian Cell Micronucleus Test

The clastogenic and aneugenic potential of 6'-SL 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, 2016a) (Phipps *et al.,* 2019a).

An initial preliminary cytotoxicity test was conducted using 6'-SL at concentrations up to 2,000 µg/mL (the OECD 487 guideline maximum recommended concentration), in the presence (3-hour treatment) and absence (3- and 24-hour treatments) of S9 metabolic activation; no cytotoxicity was observed at any dose level. Cytotoxicity was assessed again in the main experiment and again 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 6'-SL at 500, 1,000, or 2,000 µg/mL with S9 (3 hours) and without S9 (3- and 24-hour treatments). The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronucleated binucleated cells (MNBC), with the frequency of MNBC also being above upper historical vehicle control limit.

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 statistically significant and biologically relevant increases in MNBC (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that 6'-SL is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL (the OECD 487 guideline maximum recommended concentration), in the absence and presence of metabolic activation.

6.4.2 Studies Conducted with 6'-SL Produced by Other Methods

6.4.2.1 Acute Toxicity Study in Rats

A single dose acute toxicity study was conducted to evaluate the potential acute toxicity of GeneChem's 6'-SL produced by enzymatic synthesis (Gurung *et al*., 2018a). The study was conducted in compliance with GLP and according to FDA Redbook IV.C.2 (U.S. FDA, 1993).

Groups of 5 male and 5 female Sprague Dawley rats received a single dose of 0 (purified water), 5,000, 10,000, 15,000, or 20,000 mg/kg body weight/day 6'-SL by oral gavage. Animals were observed for 14 days after dosing to monitor clinical signs, body weight and food and water consumption. After the 14-day observation period a gross macroscopic necropsy was performed and selected organs were examined microscopically.

There were no deaths and no test item-related clinical signs. Body weight, organs weights and food and water consumption were similar across all groups. There were also no test item-related macroscopic or microscopic findings.

In the absence of any test item-related findings, the median lethal dose (LD_{50}) of 6'-SL was found to be greater than 20,000 mg/kg body weight/day (the highest dose tested), indicating that it is non-toxic.

6.4.2.2 90-Day Toxicity Study in Rats

A 13-week repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of GeneChem's 6'-SL produced by enzymatic synthesis (Gurung *et al.,* 2018a). The study was conducted in compliance with GLP and according to FDA Redbook IV.C.4.a (U.S. FDA, 2003a).

Groups of 11 male and 11 female Sprague Dawley rats received 0 (purified water), 1,000, 2,500, or 5,000 mg/kg body weight/day of 6'-SL, by oral gavage once daily for 13 weeks. Animals were examined twice daily from the start of treatment. Body weights and food intake were recorded weekly until necropsy. The eyes of all animals were examined before dosing and, for controls and high-dose animals only, again in Week 13. Blood samples were taken for hematology, blood chemistry and coagulation during Week 13. Urine samples were collected for urinalysis in Week 13.

All animals were subjected to a gross macroscopic necropsy at the end of the dosing period, where selected organs (liver, kidneys, adrenals, spleen, heart, lung, uterus, ovaries, testes, epididymides, thymus, brain and thyroid/parathyroid) were weighed and fixed. At the end of the treatment period, a full list of tissues (adrenal glands, femur, eyes, vagina, aorta, bone marrow, brain, cecum, colon, uterus, duodenum, epididymis, esophagus, heart, ileum, jejunum, kidneys, liver, lung, mandibular lymph nodes, mesenteric lymph nodes, mammary glands, nasal turbinates, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, spinal cord, spleen, stomach, testes, thymus, thyroid/parathyroid, trachea, and urinary bladder) for animals in the control and high-dose 6'-SL groups, were examined microscopically.

There were no test item-related differences in hematology, blood chemistry, coagulation or urinalysis parameters between 6'-SL groups and controls. Where statistically significant differences were observed [increased total protein for mid-dose females and reduced total protein for mid-dose males; increased urea for low-dose females; reduced chloride for mid-dose females; reduced alkaline phosphatase (ALP) for males given 5,000 mg/kg body weight/day; reduced globulin for mid-dose males; reduced cholesterol for low-dose males; reduced RBC for high-dose females; increased platelets for mid-dose females; reduced corpuscular hemoglobin concentration (MCHC) for low-dose males and females; increased mean corpuscular volume (MCV) for mid-dose males; increased mean corpuscular hemoglobin (MCH) for high-dose males] were inconsistent between the sexes [reduced creatinine for low-dose females; increased sodium for low-dose males; reduced white blood cells (WBC) for low-dose males] and were therefore considered unrelated to 6'-SL administration. Furthermore, all values were within historical control ranges, thus reflecting normal biological variation.

Organ weights were also unaffected by 6'-SL administration. The only statistically significant differences observed (reduced absolute spleen weight for low-dose females, reduced absolute and body weight-relative adrenal weight for mid-dose males) were not associated with a dose-response and were clearly not test item-related. Any macroscopic and microscopic findings observed were incidental and unrelated to administration of 6'-SL.

In the absence of any test item-related adverse findings, 5,000 mg/kg body weight/day (the highest dose tested) was considered the NOAEL.

6.4.2.3 Genotoxicity Studies

6.4.2.3.1 Bacterial Reverse Mutation Test

A bacterial reverse mutation test was conducted in accordance with FDA Redbook IV.C.1.a. (U.S. FDA, 2000a) and the principles of GLP (Gurung *et al.,* 2018a). Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *S.* Typhimuriu*m* strains TA97, TA98, TA100, TA102 and TA1535, which were exposed to GeneChem's 6'-SL produced by enzymatic synthesis at concentrations of up to 5,000 µg/plate in the absence and presence of S9 metabolic activation.

No increases in the number of revertant colonies were observed after exposure to 6'-SL at any concentration, in the absence of presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in the number of revertant colonies (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that 6'-SL was non-mutagenic under the conditions of this test.

6.4.2.3.2 *In Vitro* Chromosome Aberration Test

The *in vitro* chromosome aberration test was conducted in accordance with FDA Redbook IV.C.1.b (U.S. FDA, 2003b) and the principles of GLP (Gurung *et al.,* 2018a). In the main experiment for analysis, Chinese hamster lung (CHL) cells were exposed to concentrations of GeneChem's 6'-SL produced by enzymatic synthesis at 225, 450, or 900 µg/mL with and without S9. The vehicle (saline) was used as a negative control and positive controls were also included in the absence (mitomycin C) and presence (cyclophosphamide) of metabolic activation.

There was no evidence of structural or numerical chromosomal aberrations in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in structurally aberrant cells (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that 6'-SL was neither clastogenic nor aneugenic at concentrations under the conditions of this test.

6.4.2.3.3 *In Vivo* Mammalian Erythrocyte Micronucleus Test

An *in vivo* mammalian erythrocyte micronucleus test was conducted in accordance with FDA Redbook IV.C.1.d (U.S. FDA, 2000b) and the principles of GLP (Gurung *et al.,* 2018a). Based on the results of a dose-range finding study, groups of 5 male and 5 female Kunming mice were dosed by oral gavage with purified water (vehicle control) or GeneChem's 6'-SL produced by enzymatic synthesis at 500, 1,000, or 2,000 mg/kg body weight/day on 2 successive days, approximately 18 hours apart.

A positive control group (comprised of the same number of animals) received a single 40 mg/kg oral gavage dose of cyclophosphamide. Vehicle controls and 6'-SL groups were killed either 24 or 48 hours after their final dose (positive controls were killed 24 hours after dosing only). The proportion of polychromatic erythrocytes (PCEs) to total erythrocytes was calculated for each animal by analyzing at least 200

erythrocytes. In addition, a minimum of 2,000 PCE/animal were scored for the incidence of micronucleated polychromatic erythrocytes (MNPCEs).

There were no differences in the proportion of PCEs to total erythrocytes or in the incidence of micronucleated polychromatic erythrocytes for groups given 6'-SL, compared with vehicle controls. The positive control induced statistically significant increases in the incidence of MNPCE compared with vehicle controls, which demonstrated that the test system was capable of detecting a known clastogen.

Therefore, 6'-SL was considered to be neither clastogenic nor aneugenic *in vivo*, under the conditions of this test.

6.4.3 Studies Conducted with Glycom's 3'-SL

6.4.3.1 Repeat-Dose Oral Toxicity

6.4.3.1.1 14-Day Toxicity Study in the Neonatal Rat

A 14-day repeat dose toxicity study was conducted in rats to evaluate the potential short-term toxicity of 3'-SL and select dose levels for a subsequent 90-day study (Phipps *et al*., 2019b).

Groups of 8 male and 8 female neonatal rats were dosed from PND 7 with 0 (water for irrigation), 4,000 or 5,000 mg/kg body weight/day of 3'-SL, by gavage at a dose volume of 10 mL/kg body weight, once daily for 14 days, until the day before necropsy. Doses of 3'-SL were corrected to account for "other carbohydrates" within the test article batch.

All animals were observed 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 or clinical signs. One male receiving 3'-SL at 4000 mg/kg body weight/day, was found dead at the final observation occasion on Day 14 of dosing. This animal had shown no changes in clinical condition but gained slightly less weight (2%) than the other males in this group (8 to 11%) between Days 13 and 14 of dosing. Macroscopic examination revealed no abnormalities and there was no evidence of dosing trauma. In the absence of any other deaths during the study, this premature death was considered incidental and unrelated to administration of 3'-SL. There were no biologically relevant differences in body weight between test item-treated groups and controls and no test item-related macroscopic abnormalities at necropsy.

In 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 NOAEL and a suitable high-dose for the 90-day study.

6.4.3.1.2 90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of 3'-SL when administered, by gavage, to neonatal rats from PND 7 (Phipps *et al*., 2019b). The study was conducted in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b), but was adapted by using neonatal animals (as 3'-SL is intended for use in infant formula) to consider the requirements of EFSA *Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age* (EFSA Scientific Committee, 2017), *Guidance for industry: nonclinical safety evaluation of paediatric drug products* (U.S. FDA, 2006*), Guideline on the need for non-*

clinical testing in juvenile animals of pharmaceuticals for paediatric indications (EMEA, 2008), and *the Guideline on the Nonclinical Safety Study in Juvenile Animals for Paediatric Drugs* (MHLW, 2018).

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 1,000, 3,000, or 5,000 mg/kg body weight/day 3'-SL, by oral 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 3'-SL group and identify any effects related to the general fiber-like characteristics of the reference material. Doses of 3'-SL and the reference control were corrected to account for "other carbohydrates" within the test article batches. An additional 5 males and 5 females in each group were also dosed once daily for at least 90 days and then kept un-dosed for 4 weeks, to assess the reversibility of any observed effects.

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 for blood chemistry only 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; water consumption was recorded 1 week before urine collection on each occasion.

In Weeks 11 and 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.

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

There were no test item-related deaths, clinical signs, or ocular changes. One low-dose female was euthanized on Day 74 of dosing, due to clinical signs of rapid respiration, thin build and whole-body pallor; this female also lost weight (35 g) during the preceding 3 days. Macroscopic findings for this animal included thin, clear fluid in the thoracic and abdominal cavities, an enlarged heart, a firm liver and lungs and dark discoloration of several tissues. Histopathology revealed marked, hemorrhagic necrosis of the adrenal cortex (considered to be the major factor contributing to death), hemorrhagic necrosis in the centrilobular area of the liver, inflammatory infiltrate in the liver, marked thymic necrosis and a minor increase in hematopoiesis in the spleen. This isolated death was incidental and unrelated to administration of 3'-SL.

No biologically relevant differences in body weight or food consumption between 3'-SL-treated groups and controls were observed. Statistically significantly lower mean final body weight and overall mean body weight gain for males given 5,000 mg/kg body weight/day, compared with vehicle controls, were considered to be unrelated to 3'-SL, as the differences were minor (7%) and there was clearly no evidence of a dose-response. Furthermore, the mean final body weight and overall body weight gain values for the males given 5,000 mg/kg body weight/day were almost identical (within <1%) to those for reference controls.

Administration of 3'-SL had no effect 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 dosing) or on ulna length or growth. Behavior of the animals during the in-hand and arena observations in Week 11 of dosing was unaffected by 3'-SL; forelimb grip strength and rearing counts were statistically significantly lower for females given 5,000 mg/kg body weight/day compared with vehicle controls, but there was no dose-response for either parameter and similar differences were not seen for males. The mean rearing count for low-dose females was largely affected by 2 atypically low individual values (5 rearings each) in this group (the other females in this group reared 10 to 23 times and vehicle controls reared 10 to 27 times). Morris maze performance was also unaffected by administration of 3'-SL, 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 among the groups 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, respectively).

No test item-related differences in values for hematological parameters between 3'-SL-treated groups and controls were observed at the end of the treatment period. Statistically significantly decreased hemoglobin (for low-dose males and for females given 5,000 mg/kg body weight/day) and red blood cell count (for females given 5,000 mg/kg body weight/day) compared with vehicle controls were observed at the end of the dosing period, but there was no evidence of a dose-response and the differences were minor; high dose values were also similar to those for reference controls. All individual values for these groups were within respective historical control ranges for these parameters. Neutrophil concentrations for all groups of females given 3'-SL were statistically significantly higher than vehicle control values; however, there was no dose-response, this difference was not seen for males and all individual values were within the HCD range. Prothrombin time was statistically significantly shorter for all groups of males and females given 3'-SL, compared with vehicle controls. However, there was no dose-response for either sex and there were no corresponding differences in APTT between test item-treated groups and controls. Furthermore, most of the individual values for the 3'-SL groups were within HCD ranges, indicating that values were within normal biological variation. High dose 3'-SL values were also similar to those for reference controls.

There were no test item-related differences in values for blood chemistry parameters between 3'-SL-treated groups and vehicle controls at the end of the treatment or recovery periods. Statistically significant reductions in sodium (for all male 3'-SL groups and females given 5,000 mg/kg body weight/day), total protein (males given 5,000 mg/kg body weight/day), and albumin (males given 5,000 mg/kg body weight/day), in addition to statistically significant increased A/G ratio (for all male 3'-SL groups) and creatinine (for males given 3,000 or 5,000 mg/kg body weight/day) were clearly not test item-related, as there was no dose-response relationship and/or no consistency between the sexes, with all individual values being within respective HCD ranges, indicating that individual values reflect normal biological variation. Statistically significant differences in triglycerides and urea (for both sexes given 5,000 mg/kg body weight/day) and in sodium and chloride (for males given 3,000 or 5,000 mg/kg body weight/day and

females given 5,000 mg/kg body weight/day) were also considered to be unrelated to 3'-SL administration, as all individual values were within respective HCD ranges for these parameters, therefore reflecting normal biological variation rather than any effect of the test item. At the end of the treatment-free period, statistically significant differences in parameters for which differences were not observed at the end of the treatment period, were considered biologically irrelevant and unrelated to 3'-SL administration. The only statistically significant difference observed at the end of the treatment-free period (increased A/G ratio for males given 5,000 mg/kg body weight/day), was also unrelated to the test item, as the majority (4/5) of the individual values were within the HCD range for this parameter, reflecting normal biological variation.

No test item-related or biologically relevant differences in urinalysis parameters between 3'-SL treated groups and controls were observed. Statistically significantly decreased urine volume, total protein and total creatinine, in addition to increased specific gravity, were observed for males given 5,000 mg/kg body weight/day compared with vehicle controls, but there was no evidence of a dose-response for any of the parameters and these differences were not seen for females. Urinary pH was statistically significantly increased for all 3'-SL-treated groups compared to vehicle controls, but individual values for this and the other urinary parameters were within the HCD ranges for animals of this age and strain, indicating the values were within normal biological variation. These findings were also considered biologically irrelevant and unrelated to 3'-SL administration as there was also no evidence of an alteration in kidney function, no microscopic abnormalities in urine sediment, no test item-related differences in kidney weights and no test item-related macroscopic or microscopic kidney findings observed.

There were no test item-related differences in organ weights between 3'-SL treated groups and vehicle controls at the end of the dosing or recovery periods. At the end of the treatment period, statistically significantly decreased body weight-adjusted brain, testes, and prostate weights for males given 5,000 mg/kg body weight/day and increased adjusted kidney weights in all groups of 3'-SL treated females, compared with vehicle controls, were not associated with a dose-response. Mean adjusted salivary gland weight for males given 5,000 mg/kg body weight/day was statistically significantly lower that for vehicle controls; this was considered to be a consequence of the non-dose related marginally lower absolute terminal body weight of the high-dose 3'-SL males, given that there was no statistically significant difference in absolute weight. No statistically significant differences between test item-treated groups and vehicle controls were observed at the end of the treatment-free period. As there was no effect on the pituitary-thyroid axis observed during the study, the samples collected for potential analysis of TSH, T3 and T4 were not analyzed; this is in accordance with OECD Test Guideline 407 (OECD, 2008), which the EFSA *Guidance for submission for food additive evaluations* refers to regarding modification of OECD Test Guideline 408 (OECD, 1998b) studies, to include assessment of some additional parameters that place more emphasis on endocrine-related endpoints (EFSA, 2012).

Macroscopic and microscopic findings at scheduled necropsy revealed only incidental findings in all groups that are commonly observed in Sprague-Dawley rats of this age.

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

6.4.3.2 Genotoxicity Studies

6.4.3.2.1 Bacterial Reverse Mutation Test

The potential mutagenicity of 3'-SL was evaluated 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[13] B13/14, U.S. EPA Health Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998) and FDA Redbook IV.C.1.a. (U.S. FDA, 2000a) (Phipps *et al*., 2019b).

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 exposed to 3'-SL at concentrations of up to 5,000 µg/plate (the OECD 471 guideline maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix).

Water (purified by reverse osmosis) served as the vehicle for 3'-SL 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 increases in mean revertant colony numbers of at least twice (or 3 times in the case of strains TA1535 and TA1537) that of the concurrent vehicle controls (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that 3'-SL is non-mutagenic at concentrations up to 5,000 µg/plate (the OECD 471 guideline maximum recommended concentration).

6.4.3.2.2 *In Vitro* Mammalian Cell Micronucleus Test

The clastogenic and aneugenic potential of 3'-SL 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, 2016a) (Phipps *et al*., 2019b).

An initial preliminary cytotoxicity test was conducted using 3'-SL at concentrations up to 2,000 µg/mL (the OECD 487 guideline maximum recommended concentration), in the presence (3-hour treatment) and absence (3- and 24-hour treatments) of S9 metabolic activation; there was no evidence of cytotoxicity observed at any dose level. Cytotoxicity was assessed again in the main experiment, where there was no biologically relevant indication 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 6'-SL at 250, 500, 1,000, or 2,000 µg/mL with S9 (3 hours) and without S9 (3- and 24-hour treatments). The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of MNBCs compared with vehicle controls, 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, therefore, that 3'-SL is neither clastogenic nor aneugenic

[13] Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). OJ L 142, 31.5.2008, p. 1–739.

at concentrations up to 2,000 µg/mL (the OECD 487 guideline maximum recommended concentration), in the absence and presence of metabolic activation.

6.4.4 Studies Conducted With 3'-SL Produced by Other Methods

6.4.4.1 Acute Toxicity Study in Rats

A single dose acute toxicity study was conducted to evaluate the potential acute toxicity of 3'-SL produced by enzymatic synthesis (Kim *et al*., 2018). The study was conducted in compliance with GLP and according to FDA Redbook IV.C.2 (U.S. FDA, 1993).

Groups of 5 male and 5 female Sprague Dawley rats received a single dose of 0 (purified water), 5,000, 10,000 or 15,000 or 20,000 mg/kg body weight/day 3'-SL by oral gavage. Animals were observed for 14 days after dosing to monitor clinical signs, body weight and food and water consumption. After the 14-day observation period a gross macroscopic necropsy was performed and selected organs were examined microscopically.

There were no deaths and no test item-related clinical signs. Body weight, organs weights and food and water consumption were similar across all groups. There were also no test item-related macroscopic or microscopic findings.

In the absence of any test item-related findings, the LD_{50} of 3'-SL was found to be greater than 20,000 mg/kg body weight/day (the highest dose tested), indicating that it is non-toxic.

6.4.4.2 Repeat-Dose Oral Toxicity

6.4.4.2.1 28-Day Toxicity Study in Rats

A 28-day repeat dose toxicity study was conducted to evaluate the potential subacute toxicity of 3'-SL and to aid the dose level selection for the subsequent 90-day study (Kim *et al.,* 2018). The study was conducted using internationally agreed test guidelines [OECD Test Guideline 407 and FDA Redbook IV.C.3.a. (U.S. FDA, 2003c; OECD, 2008)] and in accordance with the principles of GLP.

Groups of 10 male and 10 female Sprague Dawley rats received 0 (purified water), 500, 1,000, or 2,000 mg/kg body weight/day 3'-SL, by oral gavage once daily for 28 days. All animals were assessed for effects on clinical signs, body weight, food consumption, clinical pathology parameters and organ weights. Ophthalmic, gross macroscopic and histopathological examinations were also conducted.

No test item-related findings were observed for any of the recorded parameters. In absence of any test item-related findings, 2,000 mg/kg body weight/day (the highest dose tested) was considered the NOAEL.

6.4.4.2.2 90-Day Toxicity Study in Rats

A 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of 3'-SL (Kim *et al.,* 2018). The study was conducted using internationally agreed test guidelines [OECD Test Guideline 408 and FDA Redbook IV.C.4.a (OECD, 1998b; U.S. FDA, 2003a)] and in accordance with the principles of GLP.

Groups of 10 male and 10 female Sprague Dawley rats received 0 (purified water), 500, 1,000, or 2,000 mg/kg body weight/day of 3'-SL, by oral gavage once daily for 90 days. Animals were examined daily from the start of treatment. Body weights and food intake were recorded weekly until necropsy. The eyes of all animals were examined before dosing and for controls and high-dose animals only, again in Week 13. Blood samples were taken for hematology, blood chemistry and coagulation during Week 13. Urine samples were collected for urinalysis in Week 13.

All animals were subjected to a gross macroscopic necropsy at the end of the dosing period, where selected organs (brain, pituitary, heart, lung, liver, spleen, kidney, adrenal, testis, prostate, ovary, and uterus) were weighed and fixed. At the end of the treatment period, a full list of tissues (brain, pituitary, thyroid and parathyroid, thymus, heart, lung with bronchi, trachea, liver, spleen, kidney, adrenal, esophagus, salivary gland, submandibular, sublingual and parotid gland, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, testis, epididymis, prostate, seminal vesicle, ovary, uterus, vagina, urinary bladder, submandibular and mesenteric lymph nodes, eye and Harderian gland, mammary gland, skin, bone marrow (femur and sternum), tongue, spinal cord and gross lesions) for animals in the control and high-dose 3'-SL groups, were examined microscopically.

There were no test item-related differences in hematology, blood chemistry, coagulation or urinalysis parameters between 3'-SL groups and controls. The only statistically significant difference was a decrease in glucose for males given 1,000 or 2,000 mg/kg body weight/day, which was considered unrelated to 3'-SL administration as there was no dose-response relationship and it was not observed in females. Organ weights were also unaffected by 3'-SL administration. Any macroscopic and microscopic findings observed were incidental and unrelated to administration of 3'-SL.

In absence of any test item-related adverse findings, 2,000 mg/kg body weight/day (the highest dose tested) was considered the NOAEL.

6.4.4.2.3 Dose Escalation Single Dose Toxicity in Beagle Dogs

The single dose acute toxicity of 3' SL was further evaluated in a dose escalation study in dogs (Kim *et al.*, 2018). The study was conducted in compliance with GLP.

Either 1 or 2 Beagle dogs received a single dose of the vehicle (water) or 3'-SL at 500, 1,000, or 2,000 mg/kg body weight by oral gavage, with 4-day intervals between each dose increment. Animals were observed for effects on general condition, motor activity, automatic nerve activity and defecation at 30 minutes, and 1, 2, 4, and 6 hours after each dose. Animals were observed twice daily for 2 weeks after receiving the final dose. Body weights were recorded before each dosing occasion and at 1, 3, 7 and 14 days after the final dose.

There were no deaths and no test item-related adverse clinical signs. Transient diarrhea was observed in 2 males and 1 female after the 2,000 mg/kg body weight dose, but this was considered non-adverse.

In the absence of any test item-related adverse findings, the maximum tolerated dose (MTD) of 3'-SL was found to be greater than 2,000 mg/kg body weight (the highest dose tested).

6.4.4.2.4 Tolerability Studies in Neonatal Pigs

A feeding study was conducted to assess the tolerability of a combination of 3'-SL and 6'-SL (referred to as "sialyllactose" by the publication authors) and its effects on gastrointestinal development and bacterial colonization in neonatal piglets (Monaco *et al.*, 2018). Thirty-eight naturally-farrowed male neonatal piglets were randomly assigned to 1 of 4 groups (control, low-, mid- or high-dose of sialyllactose). Milk-based formulae contained protein (whey and casein), docosahexaenoic acid (DHA), arachidonic acid (ARA) and lactose as a base for all groups, with additional carbohydrates provided in the diet for controls [galacto-oligosaccharide (GOS) and polydextrose)] and the test item-groups (sialyllactose). Animals received their respective formula from Day 2 to Day 32/33 of age (total dosing period of just over 4 weeks).

The final formulae were intended to contain 0, 130, 380 or 760 mg sialyllactose/L; however, due to inherent sialyllactose within the formulations, the final sialyllactose contents for each group were 55, 159, 429, or 779 mg/sialyllactose/L for controls, low-, mid-, and high-dose animals, respectively. The piglets were fed at 285 mL/kg body weight/day from Days 3 to 7 of age and 325 mL/kg body weight/day from Day 8 of age onwards, resulting in sialyllactose dose concentrations of 16, 45, 122, or 222 mg/kg body weight/day from Days 3 to 7 of age and 18, 52, 139, or 253 mg/kg body weight/day from Day 8 of age onwards.

At the end of the treatment period, blood was collected for hematology and blood chemistry assessment [parameters included calcium, phosphorus, magnesium, sodium, potassium, chloride, total protein, albumin, globulin, glucose, total cholesterol, triglycerides, creatinine, urea, total bilirubin, bicarbonate, ALP, AST, gamma glutamyl transferase (GGT), creatine phosphokinase (CPK), glutamate dehydrogenase (GLDH), total blood count, red blood cells (RBC) count, hemoglobin concentration, hematocrit value, MCV, MCH, MCHC, WBC count, neutrophils, lymphocytes, prothrombin time (PT), APTT, platelet count and volume)]. At necropsy, the entire small intestine was removed and weighed, before it was segmented into the duodenum, jejunum and ileum. The segments were flushed with phosphate buffered saline (PBS) and fixed in Bouin's solution for goblet cell analyses and intestinal morphology. In addition, mucosal disaccharide activity was assessed. Colonic luminal contents and feces were collected for measurement of pH, dry matter, volatile fatty acids and microbiota.

There were no reported adverse effects and body weight gain was similar across all groups. There were no differences in intestinal length or weight between controls and sialyllactose-treated groups. No test item-related differences in clinical pathology values were observed (the only statistically significant differences that were reported were generally not associated with a dose-response and values were within historical control ranges).

<u>Consumption of up to 253 mg/kg body weight/day sialyllactose for over 4 weeks was well tolerated.</u>

The short-term tolerability of 3'-SL was assessed in a 21-day study conducted using neonatal piglets (Monaco *et al.*, 2019). A total of 24 male and 24 female neonatal (2 days old at the start of dosing) piglets were obtained from 8 different litters, comprising 3 to 12 different piglets per litter. The piglets were assigned to 1 of 4 groups (12 animals per group), receiving either the control formula (commercially-available non-medicated sow-milk replacer formula) or the same formula supplemented with 3'-SL at 140, 200, or 500 mg/L (corresponding to concentrations of 135.3, 193.3 and 483.2 mg/L, respectively). The piglets were fed at 300 mL/kg body weight/day from Days 1 to 5 of age and 360 mL/kg body weight/day from Day 6 of age onwards. Three piglets from the low-dose group were removed from the study due to showing watery diarrhea for 3 days.

Clinical signs and body weights were recorded throughout the study. Blood samples were collected for analysis of clinical chemistry, hematology and coagulation parameters on Day 8 and at the end of the study,

with urine samples (for urinalysis) collected at necropsy only. At necropsy, the spleen, stomach, kidneys, heart, lungs and liver were removed and weighed before a section of each was fixed for histopathological analysis. The intestines were also weighed, segmented and sections were fixed for microscopic analysis. Mesenteric lymph nodes, pancreas and gallbladder were collected and fixed, but not weighed.

There were no test item-related adverse effects (watery diarrhea was only observed at the lowest concentration and was thus considered unrelated to 3'-SL) and body weight gain was similar across all groups. There were no differences in intestinal length or weight between controls and 3'-SL-treated groups. No test item-related differences in clinical pathology values were observed (the only statistically significant differences that were reported were generally not associated with a dose-response and values were within historical control ranges). The only macroscopic and microscopic findings observed were considered to be incidental and unrelated to 3'-SL consumption.

Consumption of 3'-SL in formula at concentrations up to 483.2 mg/L for 21 days was well tolerated.

6.4.4.3 Genotoxicity Studies

6.4.4.3.1 Bacterial Reverse Mutation Test

The bacterial reverse mutation test was conducted using internationally agreed test guidelines [OECD Test Guideline 471 and FDA Redbook IV.C.1.a. (OECD, 1997; U.S. FDA, 2000a) and according to the principles of GLP (Kim *et al.,* 2018). 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*uvr*A (pKM101), which were exposed to 3'-SL produced by enzymatic synthesis at concentrations of up to 5,000 µg/mL in the absence and presence of S9 metabolic activation. No biologically relevant increases in the number of revertant colonies were observed after exposure to 3'-SL at any concentration, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in the number of revertant colonies (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that 3'-SL was non-mutagenic under the conditions of this test.

6.4.4.3.2 *In Vitro* Chromosome Aberration Test

The *in vitro* chromosome aberration test was conducted using internationally agreed test guidelines [OECD Test Guideline 473 and FDA Redbook IV.C.1.b (OECD, 2016b; U.S. FDA, 2003b)] and in accordance with the principles of GLP (Kim *et al.,* 2018). In the main experiment for analysis, CHL cells were exposed to concentrations of 3'-SL produced by enzymatic synthesis at up to 5,000 µg/mL with and without S9. The vehicle (saline) was used as a negative control and positive controls were also included in the absence [benzo(a)pyrene and mitomycin C] and presence (mitomycin C) of metabolic activation.

There was no evidence of structural or numerical chromosomal aberrations in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant and statistically significant increases in structurally aberrant cells (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that 3'-SL was neither clastogenic nor aneugenic at concentrations under the conditions of this test.

6.4.4.3.3 *In Vitro* Mammalian Erythrocyte Micronucleus Test

The *in vivo* mammalian erythrocyte micronucleus test was conducted using internationally agreed test guidelines [OECD Test Guideline 474 and FDA Redbook IV.C.1.d (U.S. FDA, 2000b; OECD, 2016c) and in accordance with the principles of GLP (Kim *et al.,* 2018). Based on the results of a dose-range finding study, male and female Imprinting Control Region mice were dosed by oral gavage with saline (vehicle control) or 3'-SL produced by enzymatic synthesis at 500, 1,000, or 2,000 mg/kg body weight/day over 3 consecutive days. A positive control group (comprised of the same number of animals) received a single 2 mg/kg oral gavage dose of mitomycin C. Animals were killed either 24, 48, or 72 hours after their final dose.

There were no biologically relevant differences in the proportion of polychromatic erythrocytes (PCEs) to total erythrocytes or in the incidence of micronucleated polychromatic erythrocytes for groups given 3'-SL, compared with vehicle controls (statistically significant differences observed at 500 or 2,000 mg/kg body weight/day were not associated with a dose-response relationship). The positive control induced statistically significant increases in the incidence of MNPCEs compared with vehicle controls, which demonstrated that the test system was capable of detecting a known clastogen.

Therefore, 3'-SL was considered to be neither clastogenic nor aneugenic *in vivo*, under the conditions of this test.

6.5 Human Studies

No human studies have been conducted using 6'-SL; however, human data for the structural isomer 3'-SL have been published and are discussed below.

A 4-week double-blinded, controlled, randomized clinical trial was conducted to assess the gastrointestinal tolerance and safety of 3'-SL in humans (Gurung *et al*., 2018b). The study was conducted in accordance with the principles of the Declaration of Helsinki and Korea Good Clinical Practice (KGCP) Guidelines. A total of 40 *Helicobacter pylori* positive (but otherwise healthy) male and female subjects received either 12 g of 3'-SL (n = 17) or a placebo powder (n = 23) daily in 3 servings (4 g immediately after breakfast, lunch and dinner) for 4 weeks. The demographics of each group were similar (aged 32 to 65 years old and weighing 42 to 92 kg in the 3'-SL group; aged 27 to 62 years old and weighing 46 to 96 kg in the placebo group).

Adverse events were monitored throughout the study. GI symptoms were recorded at baseline and in Week 4 using a modified version of the GI symptom rating scale (GSRS) (Svedlund *et al*., 1988); physical examinations were also conducted before the start and at the end of the study. At baseline and the end of the study, clinical pathology parameters [AST, alanine aminotransferase (ALT), ALP, bilirubin, albumin, triglycerides, total and high-density lipoprotein (HDL) cholesterol, blood urea nitrogen, creatine, glucose, white blood cells, red blood cells, hemoglobin, hematocrit, and platelets] were analyzed.

There were no statistically significant differences in adverse events, GI symptoms, physical examinations or clinical pathology values. Thus, consumption of 4 g of 3'-SL 3 times daily (total of 12 g per day) for 4 weeks was well tolerated in humans.

6.6 Allergenicity

As explained in Section 2.3.3, the high purification steps involved in the manufacture are proven to remove protein (*i.e.*, potential allergen) to a level of <0.0017% (w/w).

In addition, Glycom has assessed the allergenic potential of the recombinant proteins introduced to the *E. coli* K-12 host using the search algorithms provided by the Allergen Online tool (ver. 18B) of the University of Nebraska (FARRP, 2018). This database has been updated last on 23 March 2018 and contains sequences of 2,089 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 min. 50% sequence similarity to hint for potential allergenic potential; (ii) 80 amino acid sequence segments (sliding window) comparison with an alert limit of min. 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.

As 6'-SL is produced using milk derived lactose, food products containing 6'-SL would need to include "contains milk" on the label in accordance with the requirements of the Food Allergy, Labelling and Consumer Protection Act of 2004.

6.7 Other Considerations – Additive Dietary Intakes of 6'-SL with Other HiMOs and Resistant Oligosaccharides

While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of human-identical milk oligosaccharides (HiMOs), such as 2'-FL, DFL, LNT, LNnT, 3'-SL and 6'-SL will be used in combination to produce infant formula products that are as compositionally representative of human breast milk as possible, taking into account their natural variation. Glycom recognizes that there are known gastrointestinal tolerance issues that can develop if consumed levels of indigestible carbohydrates, such as HiMOs, are too high in sensitive populations including infants. As discussed in detail previously, in Glycom's view, GRAS uses of individual HiMOs in infant formula should be representative of levels that have been reported for human milk samples obtained from lactating women across all lactational stages considering natural variation. Consequently, the maximum level of HiMOs used in combination (*i.e.*, an additive manner) in infant formula should not exceed mean quantities of total HMOs that have been measured in pooled samples of human breast milk (Kunz et al., 1999, 2000). As discussed in Section 1.3, a concentration of 0.4 g/L of 6'-SL in infant formula will ensure that consumers of infant formula containing 6'-SL will be provided with nutritional concentrations that cover 95% of the population of infants consuming human milk regardless of the secretor status of the mother. The use of mean values from pooled milk samples across multiple studies ensures that reference values for appropriate concentrations of HiMOs are not impacted by potential artifacts of a particular analytical method, or by values from individual mothers that may be biological aberrations. In all cases where Glycom's HiMOs will be used in combination with other HiMOs, the total concentrations of HiMOs will fall within conservative means of the general population, thereby ensuring that levels provided with be of nutritional value and be safe and well tolerated. For example, the total quantities of HiMOs that could be added to infant formula based on existing and future GRAS notifications for HiMOs manufactured by Glycom (*e.g.*, GRN 815 - 2'-FL/DFL, GRN 833 - LNT, GRN 659 - LNnT, as well as 3'-SL and 6'-SL) would be 3.6 g/L. This concentration is below the maximum levels of their naturally occurring HMOs counterparts in human breast milk that have been reported for pooled human milk samples (4.7 to 6.5 g/L), regardless of lactation phase, secretor/non-secretor status of mother or term/pre-term childbirth (Table 6.7-1). Therefore, taking into account that HiMOs are identical to their naturally present counterparts in human breast milk, the total amount of the proposed intended uses singly and in combination in infant formula products should not provoke any safety of tolerability concerns in infants. In fact, the most sensitive consumer group, infants in age of 1-4 days, is exposed to the highest concentrations of HMOs, as the early milk "colostrum" contains the highest levels of HMOs. Moreover, the proposed maximum use-levels of Glycom's HiMOs (singly and in combination) are well within background exposures to non-digestible oligosaccharides in infants consuming human milk, where levels of HMOs of 25 and 12 g/L have been reported in human colostrum and mature milk samples,

respectively (Kunz *et al.,* 1999, 2000) and it is therefore apparent that infants have an inherent high tolerance for these compounds.

Glycom also recognized the possibility that the company's HiMOs may be used in combination with other non-digestible carbohydrate sources such as GOS and FOS, which have GRAS status for use in infant formula. Although Glycom is not a manufacturer of infant formula, and is therefore not in a position to comment on the levels of resistant oligosaccharides such as GOS or FOS that could be used with a HiMO, or even the likelihood that such combinations would be introduced to the market, Glycom notes that any new infant formula containing a new HiMO or new HiMO combination will be subject to the laws and implementing regulations governing infant formula under Section 412 of the Federal Food, Drug, and Cosmetic Act (21 USC §350(a)). Specifically, under Section 412(d)(1) of the 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 6'-SL with an indigestible oligosaccharide such as GOS) would be well tolerated as part of the Agency's 90-day notification procedure. Section 412 therefore ensures that any combination of HiMO whether used singularly, or on an additive basis with various HiMOs will be the subject of corroborative safety and tolerance testing in infants.

Table 6.7-1 The maximum use-levels and quantities of HiMOs (g/L) from Glycom's products portfolio as proposed and/or reported in the literature.

HiMOs	GRAS Notice	Proposed Maximum Use-Level (g/kg or g/L)	Days 1 to 4 ("colostrum")		Days 5 to 14 ("transitional milk")		Days 10 to 60 ("mature milk")		Meta-analysis by Thurl *et al.* (2017)	
			Pool	Sec	Pool	Sec	Pool	Sec	Term 0 -100 days	Preterm 0 - 60 days
2'-FL[a]	GRN 815	1.60	3.2	4.0	2.5	3.3	2.2	3.0	2.74	2.77
DFL[a]	GRN 815		0.5	0.4	0.4	0.3	0.3	0.2	0.42	0.41
LNT[a]	GRN 833	0.80	0.8	0.8	0.9	0.9	1.1	1.1	0.79	1.04
LNnT[b]	GRN 659	0.60	0.52	0.52	0.42	0.42	0.40	0.40	0.74	0.66
6'-SL	This GRN	0.40	0.54	0.54	0.45	0.45	0.39	0.39	0.64	0.66
3'-SL	To be submitted	0.20	0.23	0.23	0.16	0.16	0.27	0.27	0.19	0.29
Sum		**3.6**	**5.8**	**6.5**	**4.8**	**5.5**	**4.7**	**5.4**	**5.5**	**5.8**

Pool = pooled milk samples (assuming secretor / non-secretor milk ratio of 8/2), Sec = milk samples obtained only from secretor mothers

[a] the mean HiMOs (g/L) as reported in the corresponding GRAS Notice.

[b] the mean HiMOs (g/L) results for LNnT has been updated compared to GRN 659.

6.8 GRAS Panel Evaluation

Glycom has concluded that 6'-SL 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 6'-SL, as discussed herein, and on consensus among a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Professor Emeritus, Virginia Commonwealth University School of Medicine), Dr. Robert J. Nicolosi (Professor Emeritus, University of Massachusetts Lowell), and Dr. Ronald Kleinman (Professor, Harvard Medical School).

The GRAS Panel, convened by Glycom, independently and critically evaluated all data and information presented herein, and also concluded that 6'-SL is GRAS for use in non-exempt term infant formula and specified conventional food products, as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and evaluation of such data as it pertains to the proposed GRAS uses of 6'-SL is presented in Appendix A.

6.9 Conclusion

Based on the above data and information presented herein, Glycom has concluded that the intended uses of 6'-SL 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 6'-SL in infant formula and conventional food, who similarly concluded that the intended use of 6'-SL in infant formula and conventional food as described herein is GRAS.

6'-SL 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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GRAS Panel Evaluation of 6'-Siallylactose Sodium Salt (6'-SL) for Uses in Infant Formula and Conventional Food Products

2 May 2019

INTRODUCTION

Glycom A/S (Glycom) convened a panel of independent scientists (the "GRAS Panel"), qualified by their scientific training and relevant national and international experience in the safety evaluation of food ingredients, to conduct a critical and comprehensive assessment of data and information pertinent to the safety of the company's human-identical milk saccharide (HiMS) 6'-siallylactose sodium salt (6'-SL), produced by fermentation using a modified strain of *Escherichia coli* (*E. coli*) K-12 DH1, and to determine whether the intended uses of 6'-SL in non-exempt term infant formula and various conventional food and beverage products as described in Table A-1, would be Generally Recognized as Safe (GRAS) based on scientific procedures. The GRAS Panel, consisted of the below-signed qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. Ronald E. Kleinman (Harvard Medical School), and Dr. Robert J. Nicolosi (University of Massachusetts Lowell).

The GRAS Panel, independently and collectively, critically evaluated a comprehensive package of all publicly available scientific data and information compiled from a comprehensive search of the scientific literature performed by Glycom and presented to the GRAS Panel in a dossier titled "*GRAS Status of 6'-Siallyllactose Sodium Salt (6'-SL)*" (dated 27 March 2019), 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 6'-SL and included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, the history of consumption from human breast milk, and the safety of 6'-SL.

Following its independent and collective critical evaluation, and on the basis of scientific procedures, the GRAS Panel unanimously concluded that 6'-SL, produced by fermentation using a modified strain of *E. coli* K-12 DH1, meeting food-grade specifications and manufactured in accordance with current Good Manufacturing Practice (cGMP), is GRAS for use in non-exempt term infant formula and conventional food and beverage products as described in Table A-1. A summary of the information critically evaluated by the Panel is presented below.

SUMMARY AND BASIS FOR GRAS

6'-SL manufactured by Glycom is a purified ingredient containing primarily 6'-SL (min. 90%), lactose (max. 5%) and sialic acid (max. 2%). Glycom intends to market 6'-SL in the United States (U.S.) marketplace as a food ingredient for addition to non-exempt term infant formula and various conventional food and beverage products (Table A-1). The 6'-SL ingredient is produced by fermentation using a modified strain of *E. coli* K-12 and contains no less than 94% of HiMS[1], which is characterized by the sum of 6'-SL, lactose, and sialic acid in the final product.

[1] The term HiMS is used due to the fact that 6'-SL is typically referred to as an oligosaccharide, but lactose is a disaccharide and sialic acid a monosaccharide. All of these saccharides occur naturally in human milk.

6'-SL is an acidic trisaccharide that is comprised of N-acetylneuraminic acid (sialic acid), D-galactose, and D-glucose. The reported mean concentration of 6'-SL in pooled human breast milk ranged from 0.12 to 0.54 g/L, and concentrations as high as 1.3 g/L were reported in colostrum and mature breast-milk samples by independent investigators. 6'-SL produced by microbial fermentation is chemically and structurally identical to 6'-SL (HiMS) that is naturally present in human breast milk, as confirmed by 1D-^{1}H nuclear magnetic resonance (NMR)-spectroscopy and mass spectrometry. Therefore, 6'-SL has an established long history of safe consumption as a component of human breast milk in infants on the basis that 6'-SL manufactured by Glycom is chemically identical to 6'-SL naturally present in human breast milk.

The GRAS Panel critically reviewed details of the manufacturing process for 6'-SL. The ingredient is manufactured in compliance with cGMP and incorporates a Hazard Analysis Critical Control Point (HACCP) management system. The manufacturing process can be broadly divided into 2 stages. In Stage 1 (upstream processing), D-lactose is converted to 6'-SL by the adapted cellular metabolism of the production strain which utilizes D-glucose[2] as energy and carbon sources. The production strain is a derivative of *E. coli* K-12 DH1, which is a non-pathogenic laboratory strain with a well characterized genetic history (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996). This strain was further optimized for general oligosaccharide expression features by the introduction of several modification events related to the metabolism of various carbohydrates (identical to those described in GRN 650). The strain was genomically modified to produce 6'-SL using lactose as a substrate. The activated sugar UDP-GlcNAc is produced *de novo* from fructose-6-phosphate (Fru-6-P) in three enzymatic steps catalysed by native enzymes in the bacteria. The UDP-GlcNAc is converted to the activated sugar CMP-Neu5Ac in three enzymatic steps using the following heterologous enzymes from *Campylobacter jejuni*: CMP-Neu5AC synthetase, sialic acid synthetase, and N-acetylglucosamine-6-phosphate epimerase. Lactose is transported into the host cell by lactose permease, which serves as a substrate for the enzyme, α-2,6-sialyltransferase from *Photobacterium damsela*, which catalyzes the transfer of sialic acid from the activated sugar nucleotide (CMP-Neu5Ac) to the 6'-position of lactose, resulting in the formation of 6'-SL. The gene involved in NAD biosynthesis was deleted from the genome and subsequently provided to the cells *via* plasmid to tightly link cell survival and 6'-SL production. No antibiotic resistance genes are present, and antibiotics and inducer molecules are not used. The GRAS Panel noted that the identities of the introduced genes and their expression products (*i.e*., enzymes) are well characterized, and the introduced genes would not confer toxicogenic/pathogenic properties to the host organism. The recombinant proteins were further characterized using bioinformatic tools and are not homologous to amino acid sequences of known or putative toxins or allergens.

In Stage 2 (downstream processing), a series of purification, isolation, and concentration steps are used to generate the final high-purity 6'-SL ingredient. No solvents are used during manufacturing and 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 6'-SL released conform with the product specifications. 6'-SL produced by fermentation is chemically identical to 6'-SL in human milk from lactating women. There have been no modifications to the molecular structure of 6'-SL during the manufacturing process from that of 6'-SL that is present in human milk.

Glycom has established food-grade specifications for 6'-SL. The specifications for 6'-SL include parameters related to physical properties, purity, water, sodium and chloride content, and microbiological contaminants. The main component of the ingredient is 6'-SL (min. 90%), with lactose (max 5%) and sialic acid (max 2%). Specification limits have also been established for sodium (2.5 to 4.5%), chloride (max. 1%)

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

and degradation products such as 6'-sialyllactulose (max. 3%). Lactose and sialic acid are naturally present in human milk and as a result, an additional quality parameter of min. 94% as the sum of HiMS has been established. Specifications have been established for carbohydrate-type compounds and residual proteins originating from the fermentation and downstream purification processes. All analytical methods are internationally recognized or have been validated internally. The GRAS Panel reviewed the results from 5 batches of 6'-SL and concluded that the manufacturing process produces a consistent material in conformance with the established product specifications.

The ingredient also has been evaluated for the presence of fermentation metabolites (*i.e.,* biogenic amines, amino acids, and their metabolites), microbial endotoxins, and residual proteins, the results of which demonstrate that Glycom's 6'-SL is free from these potential contaminants at levels of toxicological concern. The results of batch analyses also confirmed the absence of heavy metals. There was no appreciable carry-over of minerals from fermentation (*i.e.,* anions, trace elements), or quantifiable levels of residual DNA, in the final ingredient.

The GRAS Panel reviewed the bulk stability data of 6'-SL as described herein under real-time conditions of 25°C and 60% relative humidity (RH), and accelerated conditions of 40°C and 75% RH over a 12 month storage period. 6'-SL was stable throughout the storage period with no appreciable changes in any organoleptic properties, degradation of 6'-SL, or changes in the impurity profile (chemical and microbiological). Stress/forced stability studies of powdered 6'-SL performed at 80°C for 28 days of storage at 2 different humidity conditions (ambient and high humidity) demonstrated negligible increases in lactose and sialic acid throughout the storage period. Slight isomerization of 6'-SL to 6'-sialyllactulose was reported that was more evident at higher humidity. The stability of 6'-SL as described herein has also been evaluated in a commercially-representative infant formula, with data supporting that 6'-SL is stable in infant formula at up to 12 months of storage.

6'-SL 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 products used by the general population (see Table A-1). The maximum use-levels in term infant formulas are proposed on the basis of providing similar levels of 6'-SL on a body weight basis to those consumed by breast-fed infants. In the U.S., food uses of 6'-SL in infant formula (*i.e*., infants up to 12 months) will provide 6'-SL at a use-level of 0.4 g/L, follow-on formula at a use-level of 0.3 g/L, infant-specific foods and foods for young children at a use-level of 0.3 g/L in ready-to-drink and reconstituted products, and up to 2.5 g/kg for products other than beverages (*e.g*., baby foods). 6'-SL is also intended for use in food and beverages targeted towards the general U.S. population (up to 0.5 g/L or 5 g/kg), and foods for special dietary use (*e.g*., meal replacement bars) at levels up to 1.0 g/L or 10 g/kg.

The GRAS Panel reviewed data related to the estimated dietary exposure to 6'-SL based on an assessment of the anticipated intake of 6'-SL as an ingredient under the intended conditions of use as described in Table A-1. The dietary intakes of the ingredient were estimated using the information from the 2013-2014 cycle of the National Health and Nutrition Examination Survey (NHANES) based on the proposed food uses and use levels of 6'-SL as described in Table A-1. The Expert Panel noted that the 2015-2016 cycle of the NHANES survey was available, however, agreed that any differences between intake outcomes from use of the 2013-2014 cycle would be insignificant. A summary of the dietary intake estimates is provided in Table 1.

Table 1 Summary of the Estimated Daily Per Kilogram Body Weight Intake of 6'-SL[a] from Proposed Food-Uses in the U.S. by Population Group (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[b]	0 to 6 months	70.5	143.0	80.1	165	88.0	151.0
Infants[b]	7 to <12 months	98.5	176.0	99.9	127	98.7	176.0
Toddlers	1 to 3	34.6	70.5	98.5	460	35.1	70.5
Children	4 to 10	13.7	28.0	98.9	980	13.8	28.2
Female Teenagers	11 to 18	5.8	13.0	94.6	568	6.2	13.2
Male Teenagers	11 to 18	7.2	14.0	98.2	569	7.3	14
Female Adults of Childbearing Age	19 to 40	5.0	11.0	92.9	819	5.4	11.4
Female Adults	19 to 64	4.9	11.3	92.9	1,752	5.3	11.6
Male Adults	19 to 64	5.1	11.9	92.7	1,518	5.5	12.5
Elderly	65 and up	4.0	10.2	92.1	906	4.4	10.3
Total Population	All ages	8.1	16.0	93.8	7,045	8.6	16.8

6'-SL = 6'-Sialyllactose; 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.

[b] Intake of 3'-SL is from formula and other foods and beverages.

The GRAS Panel critically evaluated published data and information characterizing the safety of 6'-SL. This information included a discussion of the safety of the production strain, metabolic fate of HMOs, product-specific toxicological studies on 6'-SL and its isomer 3'-SL, and an allergenicity assessment of the ingredient. The GRAS Panel noted that all HiMOs manufactured by Glycom are produced from a production organism originating from the same optimized host strain (*E. coli* DH1 MDO) and HiMOs produced by Glycom using modified strains of this lineage have been the subject of extensive toxicological testing without evidence of test article-induced toxicity. The production methods employed by Glycom are based on fermentation processes that utilize food-grade lactose as a substrate and defined carbon and nitrogen sources. The existing toxicological studies conducted with Glycom's portfolio of HiMOs produced by fermentation (*e.g.,* 2'-FL, LNnT, 2'-FL/DFL, LNT, 3'-SL, 6'-SL) support the safety of the platform strain (MDO) lineage. The introduced genetic modifications for HiMO synthesis produce a predictable pattern of metabolites and intended fermentation products that are identifiable and are not of concern for imparting unexpected pleiotropic effects to fermentation products produced from this host. Based on the established safety of the strain lineage, the compositional characterization of the production process and ingredient composition (*i.e.,* identical to human oligosaccharides), and the fact that Glycom's HiMOs will be used in infant formula at levels that are equivalent to concentrations in human milk, toxicological testing was not necessary to support the safety of the ingredient. Toxicity studies of 6'-SL and 3'-SL were therefore considered corroborative in nature. The results of product-specific toxicology studies conducted with 6'-SL and 3'-SL as discussed below further support this safety conclusion.

Information characterizing the absorption, distribution, metabolism, and excretion (ADME) of human milk oligosaccharides (HMOs) were incorporated by reference to previous GRAS evaluations for LNnT and 2'-FL (*i.e*., GRN 546, 547, 650, 659). HMOs, including 6'-SL, do not undergo any significant digestion in the upper gastrointestinal tract. Small quantities of HMOs have been reported to be absorbed intact following ingestion by infants, and approximately 1 to 2% of the ingested quantities of HiMOs are excreted unchanged in the urine. The data supports limited absorption of 6'-SL and the quantities absorbed would not be different from those occurring in breast-fed infants.

A battery of published toxicity studies, including an adapted subchronic (90-day) oral toxicity study with neonatal rats, a bacterial reverse mutation assay, and an *in vitro* mammalian cell micronucleus test in human lymphocytes were conducted using 6'-SL manufactured by Glycom. These studies were performed in accordance with Organisation for Economic and Co-operation Development (OECD) Principles of Good Laboratory Practices (GLP) and appropriate OECD Test Guidelines.

The GRAS Panel critically evaluated the results of a 14-day range-finding toxicity study and a 90-day subchronic toxicity study conducted in neonatal Crl:CD(SD) rats administered 6'-SL manufactured by Glycom by gavage at doses up to 5,000 mg/kg body weight/day (Phipps *et al.,* 2019a). No test article-related adverse findings were reported in the 14-day toxicity study, which served as the basis for the tested doses in the subsequent 90-day toxicity study. The 90-day toxicity study was conducted in accordance with OECD GLP and OECD Test Guideline 408 modified for the target population (*i.e.,* infants) considering the requirements of the European Food Safety Authority (EFSA) Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (EFSA, 2017), Guidance for industry: nonclinical safety evaluation of paediatric drug products (U.S. FDA, 2006), Guideline on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications (EMEA, 2008), and the Guideline on the Nonclinical Safety Study in Juvenile Animals for Paediatric Drugs (MHLW, 2018). In the 90-day toxicity study, neonatal Crl:CD(SD) rats (10/sex/group) were administered 6'-SL by gavage at doses of 0, 1,000, 3,000, or 5,000 mg/kg body weight/day and were examined for the standard toxicological battery (mortality, clinical signs, hematology, clinical chemistry, urinalysis, macroscopic and microscopic examination) (Phipps *et al.,* 2019a). With the exception of unilateral tubular atrophy of the testis reported in 4 male animals of the 5,000 mg/kg body weight/day group, no test article-related adverse findings were reported in any study parameter in any other group. The GRAS Panel noted that unilateral atrophy of the testes is a low-level background finding in the rat; however, the number of affected animals in the high-dose group was outside the expected background range of 1 to 2%. The unilateral nature of the findings is suggestive that the findings are not test article related as chemical induced tubular atrophy typically manifests in bilateral lesions; however, unilateral tubular atrophy has been reported on occasion for some compounds such as high doses of L-cysteine (La *et al.,* 2012). As reported by Phipps *et al.,* (2018), chemical induced unilateral atrophy is typically secondary to impaired fluid reabsorption in the efferent ducts leading to increased fluid volume and back-pressure upstream of the blockage. Dilation of the seminiferous tubules ensues, which, in its late stages, progresses to tubular atrophy. The histopathological findings in the 6'-SL treated rats were absent expected changes that accompany excurrent ductular blockage[3], which argues against this mechanism of toxicity. In addition, chemical-induced unilateral atrophy secondary to excurrent ductular blockage would be expected to be a progressive degenerative effect and the absence of any correlating histopathological changes in the lower dose groups is strongly suggestive that the finding is not test article related. The GRAS Panel also noted that no evidence of testicular toxicity was reported in other toxicity studies of 6'-SL; *e.g.,* a NOAEL of 5,000 mg/kg body weight/day, the highest dose tested, was reported for 6'-SL produced by enzymatic synthesis in a 90-day toxicity study in Sprague-Dawley rats (Gurung *et al.,* 2018a). The absence of testicular toxicity in studies conducted in mature and neonatal rats administered the 3'-SL isomer (Phipps *et al.,* 2019b; Kim *et al.,* 2018) corroborates a conclusion that the testicular findings are not test article related. The GRAS Panel concluded that the findings of unilateral tubular atrophy of the testis and absent sperm in the epididymis to be incidental and unrelated to administration of 6'-SL. Based on the results of this study, the NOAEL for 6'-SL was concluded to be 5,000 mg/kg body weight, the highest dose tested. The GRAS Panel concurs with this NOAEL.

The GRAS Panel reviewed findings from acute and subchronic toxicity studies conducted with 6'-SL manufactured by GeneChem (Daejeon, Republic of Korea) using enzymatic synthesis (Gurung *et al*., 2018a).

[3] Increased testicular weight due to fluid accumulation, chronic inflammatory or atrophic changes of the affected segment of the epididymis, rete tubular dilatation or dilation of the testicular and/or epididymal tubules due to stasis and fluid/sperm accumulation

These studies were conducted in compliance with GLP guidelines and according to OECD or FDA Redbook guidelines for the toxicity testing of chemicals and food additives. Findings from the acute toxicity investigation demonstrated that 6'-SL is of low toxicity potential and the estimated Lethal Dose 50 (LD_{50}) was >20,000 mg/kg body weight. Findings reported for the subchronic toxicity study were unremarkable. A NOAEL of 5,000 mg/kg body weight/day, the highest dose tested was reported (Gurung *et al.,* 2018a).

The GRAS Panel also reviewed published toxicity studies of 3'-SL (Phipps *et al.,* 2019b; Kim *et al.,* 2018). Based on the chemical and structural similarity between 6'-SL and 3'-SL, the GRAS Panel considered studies of 3'-SL to be relevant to the safety evaluation of 6'-SL. Toxicity studies of 3'-SL manufactured by Glycom were conducted using the same doses and study design incorporating neonatal rats as was previously reported for 6'-SL (Phipps *et al.,* 2019a). The gavage administration of 3'-SL at doses of up to 5,000 mg/kg body weight/day for 90 days was unremarkable. Based on the absence of test article related findings in this study, the NOAEL for 3'-SL was concluded to be 5,000 mg/kg body weight/day, the highest dose tested. Similar results reported for 3'-SL produced by enzymatic synthesis further corroborate these findings (Kim *et al.,* 2018).

The GRAS Panel also critically evaluated the results of genotoxicity and mutagenicity bioassays conducted with Glycom's 6'-SL and 3'-SL ingredients. The potential mutagenicity of these HiMOs was evaluated in the bacterial reverse mutation test using *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were treated with 6'-SL or 3'-SL at concentrations of up to 5,000 µg/plate in the presence and absence of metabolic activation. The potential genotoxicity of 6'-SL and 3'-SL was evaluated in an *in vitro* mammalian cell micronucleus test conducted using human lymphocytes at concentrations of 500, 1,000, or 2,000 µg/mL with S9 (3 hours) and without S9 (3- and 24-hour treatments). Both bioassays were performed in accordance with respective OECD Test Guidelines and Principles of GLP. Similar studies performed with 6'-SL and 3'-SL produced by enzymatic synthesis (Kim *et al.,* 2018; Gurung *et al.,* 2018a) provide further evidence that Glycom's 6'-SL and 3'-SL, like other HMOs that are natural constituents of human breast milk, are non-mutagenic, non-clastogenic, and non-aneugenic, and that the manufacturing process did not introduce undesirable substances with potential mutagenic/genotoxic potential.

Tolerability studies were conducted in neonatal pigs (Monaco *et al.,* 2018, 2019) and the results demonstrate that "sialyllactose" (combination of 3'-SL and 6'-SL) and 3'-SL was well tolerated in the diet at concentrations up to 483.2 mg/L for 21 days.

No clinical studies on 6'-SL were identified in the literature. Human data for the structural isomer 3'-SL that included a 4-week double-blinded, controlled, randomized clinical trial evaluating the gastrointestinal tolerance and safety of 3'-SL were reported by Gurung *et al.* (2018b). Forty *Helicobacter pylori* positive, but otherwise healthy, male and female subjects consumed 12 g of 3'-SL (n=17) or placebo (n=23) daily in 3 servings for 4 weeks. No significant differences in adverse events, gastrointestinal symptoms, physical examinations, or clinical pathology were reported, and it was concluded that consumption of 12 g of 3'-SL daily (4 g in 3 servings) for 4 weeks was well tolerated in humans.

The allergenic potential of the recombinant proteins expressed by the production strain was assessed using bioinformatic analyses. The amino acid sequences of the recombinant proteins were evaluated using the BLAST search algorithms of the AllergenOnline database (version 18B) of the Food Allergen Research and Resource Program (FARRP) of the University of Nebraska (FARRP, 2018). 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; and (iii) 8 mer sequence segments (sliding

window) with an alert limit of full match to hint for potential allergenic potential. No sequence alerts for potential allergenicity were identified. In addition, the purification steps involved in the manufacture of 6'-SL 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 GRAS Panel considered the risk of allergenicity to be very low. The GRAS Panel noted that since lactose is used as a substrate during fermentation that food products containing 6'-SL 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 6'-SL, including preclinical and clinical studies, the GRAS Panel unanimously concluded that the available information supports the conclusion presented on the next page.

CONCLUSION

We, the GRAS Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that 6'-sialyllactose sodium salt (6'-SL), 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.

(b) (6)

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ATTACHMENT A1: INTENDED FOOD USES AND USE-LEVELS FOR 6'-SL IN THE UNITED STATES

Table A-1 Summary of the Individual Proposed Food Uses and Use-Levels for 6'-SL 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	0.24	1
	Sports and Isotonic Drinks, Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	360 mL	0.18	0.5
Infant and Toddler Foods	Non-exempt Term Infant Formulas	100 mL[c]	0.04	0.4
	Toddler Formulas	100 mL[c]	0.03	0.3
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.02 to 0.42	2.5
	Other Drinks for Young Children	120 mL	0.04	0.3
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	0.4	10
	Cereal and Granola Bars	40 g	0.2	5
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk*	240 mL	0.12	0.5
Milk Products	Buttermilk*	240 mL	0.12	0.5
	Flavored Milk	240 mL	0.12	0.5
	Milk-Based Meal Replacement Beverages, for Weight Reduction[b]	240 mL	0.24	1
	Yogurt*	170 g	0.86	5

6'-SL = 6'-Sialyllactose; 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.
*6'-SL is intended for use in unstandardized products when standards of identity do not permit its addition.