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

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

PREPARED BY:
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DATE:
30 June 2021

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

TABLE OF CONTENTS

List of Figures and Tables

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

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR § 170 Subpart E consisting of § 170.203 through 170.285, Inbiose N.V. (Inbiose) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of 6'-sialyllactose (6'-SL) sodium salt, as manufactured by Inbiose, in non-exempt term infant formula and various conventional food and beverage products as described in Section 1.3 below, are not subject to the premarket approval requirements of the *Federal Food, Drug, and Cosmetic Act* based on Inbiose's view that these notified uses of 6'-SL sodium salt are Generally Recognized as Safe (GRAS). To the best of our knowledge the data and information presented in this Notice represent a complete and balanced submission that is representative of the generally available literature. Inbiose considered all unfavorable as well as favorable information that is publicly available and/or known to Inbiose and that is pertinent to the evaluation of the safety and GRAS status of 6'-SL sodium salt as a food ingredient for addition to non-exempt term infant formula and various conventional food and beverage products, as described herein.

Signed,

Joeri Beauprez, PhD
Chief Scientific Officer (CSO)

30 June 2021

Date

1.1 Name and Address of Notifier

Inbiose N.V.
Technologiepark Zwijnaarde 82 – bus 41
B-9052 Gent
Belgium

1.2 Common Name of Notified Substance

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

1.3 Conditions of Use

Inbiose's 6'-SL sodium salt is proposed for use as an ingredient in non-exempt term infant formula products and a variety of other food and beverage products, as described in a previous GRAS Notice (see Table 1.3-1 from GRN 881; U.S. FDA, 2020a). All food uses of 6'-SL sodium salt produced by Inbiose will be substitutional to other GRAS sources of 6'-SL (*e.g.*, GRN 881, 922), and therefore additive increases in 6'-SL consumption are not expected to occur (U.S. FDA, 2020a, 2021).

A summary of the proposed food categories and use levels of 6'-SL, as described in GRN 881, is provided in Table 1.3-1 below (U.S. FDA, 2020a). 6'-SL sodium salt manufactured by Inbiose will be adjusted based on purity to provide the same use levels of 6'-SL as those described in GRN 881 (U.S. FDA, 2020a).

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for 6'-SL in the U.S. (Adapted from GRN 881) (U.S. FDA, 2020a)

Food Category (21 CFR § 170.3) (U.S. FDA, 2020b)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level (g/RACC)[d]	Proposed Maximum Use Level (g/kg or g/L)[d]
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; CFR = *Code of Federal Regulations*; GRN = GRAS Notice; RACC = Reference Amounts Customarily Consumed per Eating Occasion; U.S. = United States.

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

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

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

[d] Use levels expressed as quantity of 6'-SL

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

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2020d), Inbiose 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 Notice will be sent to the U.S. FDA upon request, or will be available for review and copying at reasonable times at the offices of:

Inbiose N.V.
Technologiepark Zwijnaarde 82 – bus 41
B-9052 Gent
Belgium

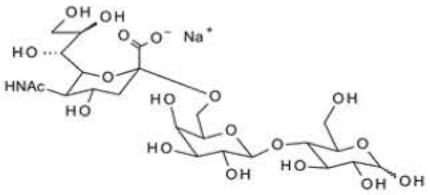
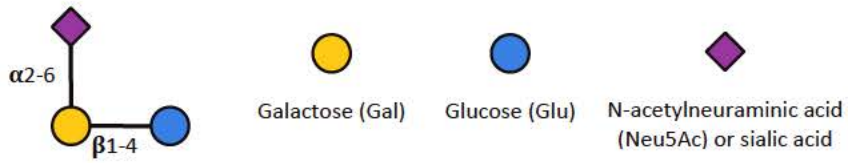
Should the FDA have any questions or additional information requests regarding this Notice, Inbiose will supply these data and information upon request.

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

It is Inbiose'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

Common Name:	6'-Sialyllactose (6'-SL) sodium salt
Abbreviation:	6'-SL (6'SL, 6-SL, 6SL) sodium salt
International Union of Pure and Applied Chemistry (IUPAC) Name:	*N*-acetyl α-neuraminic-(2→6)-β-D-galactose-(1→4)-D-glucose, sodium salt
Chemical Abstracts Service (CAS) Number:	157574-76-0
Chemical Formula:	$C_{23}H_{38}NO_{19}Na$
Molecular Weight:	655.53 g/mol
Chemical Structure:	
Schematic Representation:	

2.1.1 Chemical and Physical Characteristics

6'-SL is an abundant human milk oligosaccharide (HMO), comprised of galactose, glucose, and sialic acid.

Inbiose's 6'-SL sodium salt is produced by fermentation with a genetically modified strain of *Escherichia coli* K-12 MG1655. The final product is a purified white powder containing ≥88% 6'-SL sodium salt, and small quantities of lactose, sialic acid, and other related carbohydrates.

The identity of Inbiose's 6'-SL sodium salt has been confirmed by nuclear magnetic resonance (NMR), by comparison with a 6'-SL sodium salt reference standard (IsoSep AB, Sweden) derived from human milk. Both 6'-SL sodium salt spectra were identical and in agreement with 6'-SL ^{1}H NMR spectra from the literature (Kjærulff, 2014).

2.2 Manufacturing

2.2.1 Production Microorganism

2.2.1.1 Host Organism

The host organism is *Escherichia coli* K-12 strain MG1655, which is the same host organism as described in GRN 749, 897, and 951 (U.S. FDA, 2018a, 2020e,f). The taxonomy of the species is as follows:

Bacteria
Proteobacteria
Gammaproteobacteria
Enterobacteriales
Enterobacteriaceae
Escherichia
Escherichia coli
Escherichia coli K-12

The host strain, *E. coli* K-12 strain MG1655 is available from both American Type Culture Collection (ATCC) as 700926 and the Coli Genetic Stock Center as CGSC#7740. *E. coli* strains proliferate *via* asexual reproduction. This strain is nonrecombinant, stable, and can easily be maintained as a homogeneous population under the usual laboratory and production conditions. This strain does not produce spores.

E. coli K-12 strain MG1655 is derived from the well-known *E. coli* K-12 strain *via* classical, nonrecombinant genetics and cured of the temperate bacteriophage lambda and F plasmid by means of ultraviolet light and acridine orange, respectively. The genotype of the recipient microorganism is F-lambda-ilvG-rfb-50 rph-1, and the serotype is IRLH48:K- (Blattner *et al*., 1997). Later additional mutations in commonly used stocks of *E.coli* K-12 strain MG1655 were identified and determined to cause loss of function of the *glpR* and *crl* genes, which are involved in glycerol 3-phosphate and RNA polymerase formation, respectively (Freddolino *et al*., 2012). The complete genome of this strain has been sequenced (GenBank U00096[1]).

The United States Environmental Protection Agency conducted a risk assessment of *E. coli* K-12 under the *Toxic Substances Control Act* (U.S. EPA, 1997). This review concluded that "*the use of E. coli K-12 under contained conditions in fermentation facilities*" will present a low risk of release of this microorganism to the environment and would not pose any significant ecological hazards, based on the following evidence:

1. Wild-type *E. coli* is an inhabitant of the human colon.

2. Studies have demonstrated that *E. coli* K-12 is a debilitated strain, defective in at least 3 cell wall characteristics that are important for colonization. As a result, *E. coli* K-12 is unable to colonize the human intestinal tract under normal conditions. Even in germ free mice, *E. coli* K-12 is a poor colonizer.

3. Evidence indicates indigenous intestinal microorganisms have a large competitive advantage over *E. coli* K-12 strains.

[1] https://www.ncbi.nlm.nih.gov/nuccore/545778205/.

4. *E. coli* K-12 lacks the ability to produce significant quantities of toxins that affect humans. There is no record in the literature of *E. coli* K-12 enterotoxin-induced disease in fermentation workers.

5. *E. coli* K-12 has a history of safe commercial use. Its derivative strains are currently used in a large number of industrial applications, including the production of specialty substances L-aspartic, inosinic, and adenylic acids, which the human body produces, and U.S. FDA-approved human drugs such as insulin and somatostatin.

Because *E. coli* K-12 is not considered a human or animal pathogen and is not toxicogenic it falls into Biosafety Level 1 classification and meets the Organisation for Economic Co-operation and Development (OECD) Good Industrial Large-Scale Practice (GILSP) criteria (OECD, 1992). *E. coli* K-12 strain MG1655 has been classified Biosafety Level 1 by the ATCC[2].

2.2.1.2 Production Strain

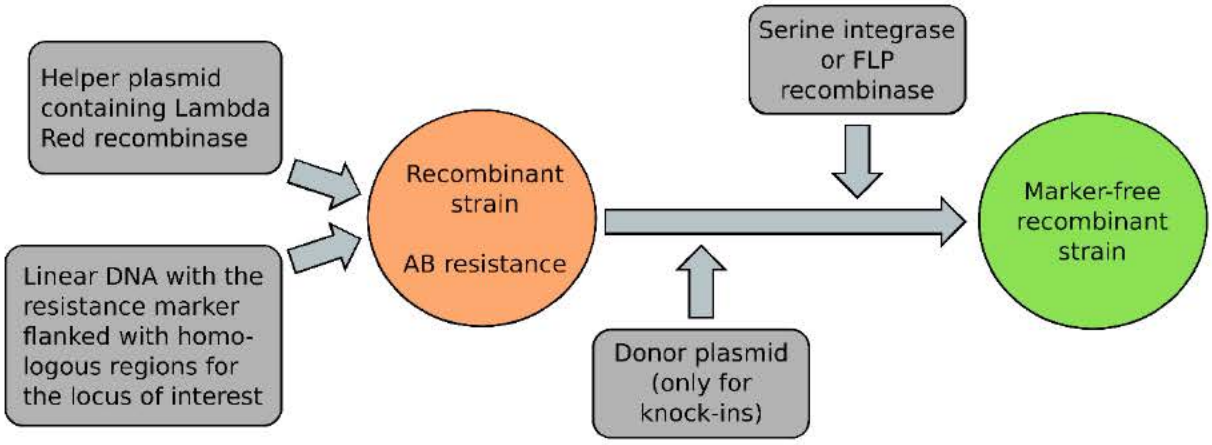
Several modifications, like gene knock-outs, gene insertions, and the addition of a production plasmid, were performed in *E. coli* K-12 strain MG1655 to create the 6'-SL production strains. Two production strains have been developed: Strain INB-6SL_01 and Strain INB-6SL_02. Strain INB-6SL_01 was the first-generation strain and 6'-SL produced from this strain has been fully characterized analytically and also has been evaluated in toxicological studies. Strain INB-6SL_02 is an optimized variant derived from the same strain lineage as Strain INB-6SL_01 and displays greater productivity relative to the first-generation strain. Both strains have been characterized genotypically and phenotypically, are stable, and produce a 6'-SL product that meets the same specifications and purity/impurity profiles. Only Strain INB-6SL_02 will be utilized commercially. Analytical data presented in Section 2.3.2 demonstrate that 6'-SL produced from either strain are chemically equivalent.

The general method to introduce genetic modifications like gene deletions and gene knock-ins into the production strain genome is based on the methods described in detail by Datsenko and Wanner (2000) and Snoeck *et al*. (2019). The method is briefly described below in Figure 2.2.1.2-1. In all cases, gene deletions and gene insertions were verified by polymerase chain reaction (PCR), Sanger sequencing, and whole genome sequencing (WGS). As validated through WGS, the final strain does not contain any trace of (i) helper plasmids; (ii) antibiotic markers present on the helper plasmids; (iii) or antibiotic markers inserted into the genome. The removal of the helper plasmid is also validated by (i) PCR and (ii) replica plating on a plate containing the antibiotic for which the marker is present on the helper plasmid. In the case of the PCR test, no amplification was observed when the plasmid was not present; in the case of the replica plate, no growth was observed for the strains that did not contain the helper plasmid.

In most cases, DNA scars (att or FRT sites) are left behind, although very small and far apart in the chromosome. Inbiose's host requires an external recombinase to recombine DNA fragments efficiently. The endogenous system requires very large stretches of homology, which are not present in the production host, and is very inefficient. After each modification, each of the previous modifications were checked by PCR and Sanger sequencing to ensure no other modifications occurred during the engineering process. No additional modifications or chromosome re-arrangements were observed, which was validated with WGS.

[2] https://www.atcc.org/~/ps/47076.ashx.

Figure 2.2.1.2-1 General Scheme of the Strain Construction Process*



* At the end after plasmid curing, a complete marker-free recombinant strain is obtained. Helper plasmids used contain a lambda Red recombinase for homologous recombination or a serine integrase recognizing att sites or a FLP recombinase recognizing FRT sites. For genomic knock-ins, an extra donor plasmid containing (heterologous) genes, flanked by att sites, needs to be added.

All heterologous genes introduced into INB-6SL_01 and INB-6SL_02 were produced by DNA synthesis and were based on well-known annotated genomes from the respective donor organism. As such, no PCR techniques were used, indicating that there is no risk of undesirable or unintended genes from the donor organism being introduced to the production host. If needed, the heterologous genes were codon-optimized using bio-informatic tools. Also, before and after introducing these heterologous genes into the genome of the production host organism, a full Sanger sequencing of the transcription units was performed to ensure their identity.

The host organism *E. coli* K-12 strain MG1655 was modified by genomic knock-outs and knock-ins by using the methods as described above to obtain efficient biosynthesis of 6'-SL (see Figure 2.2.1.2-1 above and Table 2.2.1.2-1 below).

Table 2.2.1.2-1 Genetic Modification of the Production Organisms (Gene Knock-ins)

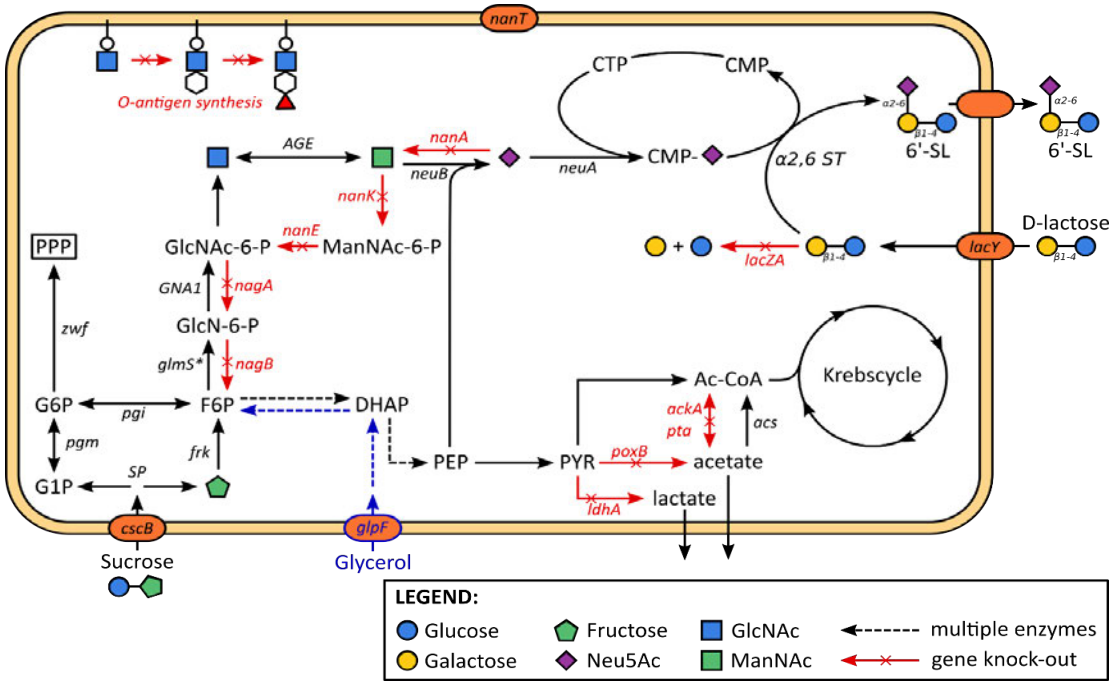
Origin	Function	Gene Modification	
		Production Strain INB-6SL_01	Production Strain INB-6SL_02
Escherichia coli	Lactose permease	Present	Present
Escherichia coli	Glutamine-fructose-6-phosphate aminotransferase	Present	Present
Saccharomyces cerevisiae	Glucosamine 6-phosphate *N*-acetyltransferase	Present	Present
Campylobacter jejuni	Sialic acid synthase	Present	Absent
Neisseria meningitidis	Sialic acid synthase	Absent	Present
Bacteroides ovatus	*N*-acylglucosamine 2-epimerase	Present	Present
Campylobacter jejuni	*N*-acylneuraminate cytidylyltransferase	Present	Present
Haemophilus influenzae	*N*-acylneuraminate cytidylyltransferase	Present	Present
Photobacterium damselae	Sialyltransferase	Present	Present
Escherichia coli	*N*-acetylneuraminate transporter	Present	Present
Escherichia coli	Sucrose permease	Absent	Present
Bifidobacterium adolescentis	Sucrose phosphorylase	Absent	Present

Table 2.2.1.2-1 Genetic Modification of the Production Organisms (Gene Knock-ins)

Origin	Function	Gene Modification	
		Production Strain INB-6SL_01	Production Strain INB-6SL_02
Zymomonas mobilis	Fructokinase	Absent	Present
Escherichia coli	Acetyl-coenzyme A synthetase	Absent	Present

In the first instance, a first-generation strain INB-6SL_01 was constructed. Knock-outs were performed to avoid the breakdown of lactose and sialic acid and to prevent the by-product formation of acetate and lactate (see Figure 2.2.1.2-2). This strain was further modified to biosynthesize 6'-SL by the introduction of genes throughout the genome (see Table 2.2.1.2-1 and Figure 2.2.1.2-2). Strain INB-6SL_01 was further modified towards the second-generation production strain INB-6SL_02. An additional knock-out disrupting multiple genes of the O-antigen synthesis pathway was performed. This strain was also further modified with extra gene knock-ins to enhance 6'-SL biosynthesis, further reduce acetate accumulation, and make strain INB-6SL_02 grow on sucrose as the carbon source (see Table 2.2.1.2-1 and Figure 2.2.1.2-2). No significant differences in carbon metabolism exist between the 2 strains; only the route towards fructose-6-phosphate and the entrance to the sialic acid production pathway differ when growing with either glycerol or sucrose carbon sources (see Figure 2.2.1.2-2). In addition to the chromosomal modifications, a plasmid was introduced in both production hosts INB-6SL_01 and INB-6SL_02 for overexpression of a *Pasteurella multocida N*-acylneuraminate cytidylyltransferase and a *Photobacterium damselae* sialyltransferase gene. No antibiotic resistance genes are present on the plasmid. The whole vector was synthesized *de novo* and was named pINB-6SL_01. After strain construction, WGS and colony PCR checks were performed to verify all genetic modifications introduced in the 6'-SL production strains. Production strain INB-6SL_01 and INB-6SL_02 do not contain any antibiotic resistance marker on the plasmid or introduced inside its genome.

Figure 2.2.1.2-2 Schematic Overview of the 6'-SL Biosynthetic Pathway in INB-6SL_01 (using glycerol as carbon source, indicated in blue) and INB-6SL_02 (using sucrose as carbon source)*



*A knock-out of the O-antigen synthesis was not present in INB-6SL_01, but was performed in INB-6SL_02.
6'-SL = 6'-sialyllactose; Ac-CoA = acetyl-coenzyme A; CMP = cytidine monophosphate; CTP = cytidine triphosphate; DHAP = dihydroxyacetone phosphate; F6P = fructose-6-phosphate; G1P = glucose-1-phosphate; G6P = glucose-6-phosphate; GlcN-6-P = glucosamine-6-phosphate; GlcNAc-6-P = N-acetylglucosamine-6-phosphate; ManNAc-6-P = N-acetylmannosamine-6-phosphate; PEP = phosphoenolpyruvate; PPP = pentose phosphate pathway; PYR = pyruvate.

Taxonomical verification was performed with FastANI[3]. Assembled contigs of the production strain were compared to *E. coli* K-12 MG1655 (U00096.3) reference genome. A whole-genome average nucleotide identity (ANI) of >99.95 was obtained confirming that the production strain is *E. coli* K12 MG1655.

Both production strains INB-6SL_01 and INB-6SL_02 proved to be 100% stable within the production environment after analysis by next generation sequencing of samples at the end of fermentation at pilot scale. The genes integrated into the genome cannot be mobilized or transferred by vector mediated processes such as conjugation. There are no known lytic phages or conjugation plasmids in these host strain; therefore, transfer can only occur by natural transformation. The integrated genes can be transferred at a frequency normal for chromosomal genes.

The final strains INB-6SL_01 and INB-6SL_02 do not contain any trace of helper plasmids used for strain construction, or from the antibiotic marker used in the construction of the helper plasmids. Some DNA scars are left in the genome after constructing gene knock-outs or gene insertions. The removal of helper plasmids is validated by PCR and replica plating on a plate containing the antibiotic for which the marker is present on the helper plasmid.

[3] https://github.com/ParBLiSS/FastANI

No specific toxic or allergenic effects are expected from the proteins expressed by the introduced genes (see Section 6.5). These proteins are not secreted, and the cell mass is separated from the product during manufacturing. The absence of these substances has been confirmed in the product specification and batch analyses.

The production process of 6'-SL with INB-6SL_01 and INB-6SL_02 does not require the addition of any antibiotics or inducer molecules. During the fermentation process, the production strains remain intact and convert their carbon source (glycerol or sucrose) into 6'-SL, which is partly secreted into the medium. Afterwards, the remaining intracellular 6'-SL is released after pasteurization. Finally, all remaining biomass of the production hosts is removed *via* a series of downstream processing steps. As such, both production hosts are solely used as a processing aid for 6'-SL biosynthesis and cannot be found in the final product.

Both production strains INB-6SL_01 and INB-6SL_02 were deposited in an internationally recognized culture collection having acquired the status of International Depository Authority under the Budapest Treaty in Belgium.

More specifically, the strain INB-6SL_01 with deposition number LMBP 12505 and strain INB-6SL_02 with deposition number LMBP 12506 were deposited at:

Belgian Co-ordinated Collections of Micro-organisms (BCCM)
GeneCorner Plasmid Collection
Ghent University - Department of Biomedical Molecular Biology
Technologiepark-Zwijnaarde 71
9052 Gent
BELGIUM

2.2.2 Raw Materials, Processing Aids, and Equipment Specifications

6'-SL sodium salt is manufactured by Inbiose in compliance with current Good Manufacturing Practice (cGMP), principles of Hazard Analysis and Critical Control Points (HACCP) and Food Safety System Certification (FSSC) 22000. The manufacture of 6'-SL is largely comparable to the production processes previously evaluated for other HMOs produced by microbial fermentation involving the construction of a production organism engineered to synthesize human-identical milk oligosaccharides (HiMOs) from lactose, large-scale fermentation and downstream processing to isolate the HiMO. All of these processes are fermentative based processes for HMOs, similar or the same as 6'-SL sodium salt. All additives, processing aids, and food contact articles used during manufacturing are permitted by federal regulation, have been previously concluded to be GRAS for their respective uses, or have been the subject of an effective food contact notification.

2.2.3 6'-SL Manufacturing Process

In summary, the manufacturing method for 6'-SL entails a fermentation process with a K-12-based production host (see Section 2.2.1) that produces 6'-SL sodium salt. This host produces 6'-SL sodium salt through the utilization of a carbon source, glycerol, and glucose or sucrose in combination with lactose in a minimal medium. The product is secreted into the medium, where it then undergoes purification before drying to produce the final dry powder product.

In the first step, biomass is removed together with cell components and large molecules (DNA, protein and lipopolysaccharides). After removal of the larger particles, the salts present in the medium are largely removed, which are cations (*e.g.,* magnesium, calcium, ammonium,) and anions (*e.g.,* phosphate and sulfate, which are minerals used for growth of the microorganism), followed by the removal of color components. The product is also converted fully in the sodium form in these steps. Water is mainly removed from the product through evaporation. Before drying the product is filtered again to ensure compliance to the microbial specifications. Figures 2.2.3-1 and 2.2.3-2 below depict the fermentation and purification processes, respectively.

Figure 2.2.3-1 Fermentation Process

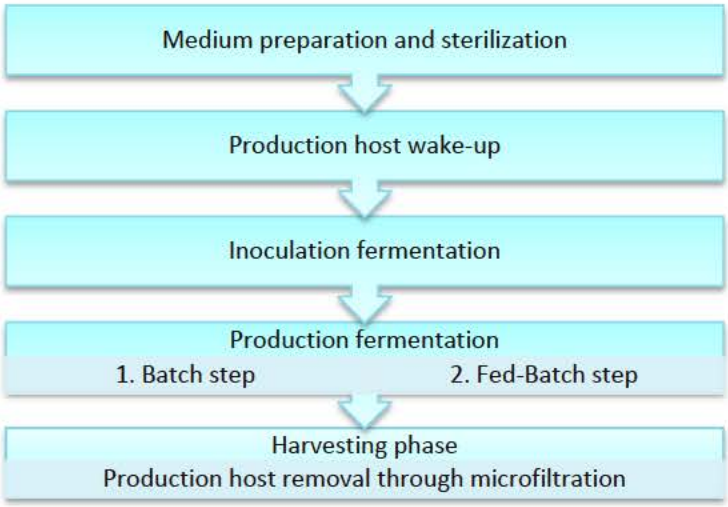
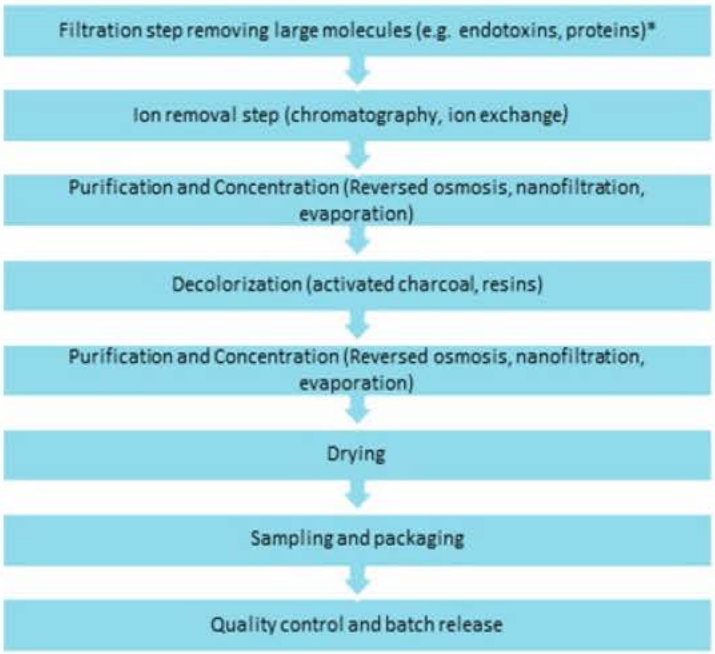


Figure 2.2.3-2 Purification Process



* The filtrations steps are done with cut-offs of 0.1 µm to 5 µm and 1 to 30 kDa.

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

To ensure consistent product quality, Inbiose has established a set of specifications for 6'-SL sodium salt, which includes the amount of 6'-SL and other main carbohydrates, chemical parameters, heavy metals, microbial contaminants, and absence of the genetically modified production strain and endotoxins. The specifications proposed for 6'-SL sodium salt are presented in Table 2.3.1-1. The specifications of Glycom A/S's (Glycom's) and Jennewein Biotechnologie GmbH's (Jennewein's) 6'-SL sodium salt (GRN 881 and GRN 922, respectively) are included in the table for comparison (Glycom A/S, 2019a; Jennewein Biotechnologie GmbH, 2020a; U.S. FDA, 2020a, 2021). All parameters were determined using compendial or validated methods.

Table 2.3.1-1 Product Specifications for Inbiose's 6'-SL Sodium Salt in Comparison to the 6'-SL Ingredient in GRN 881 and 922 (U.S. FDA, 2020a, 2021)

Parameter	Specification for Inbiose's 6'-SL	Method of Analysis Employed by Inbiose	Glycom's 6'-SL Sodium Salt (GRN 881) (U.S. FDA, 2020a)	Jennewein Biotechnologie's 6'-SL Sodium Salt (GRN 922) (U.S. FDA, 2021)
Identification				
Appearance (color)	White	Visual	White to off-white	White to ivory-colored
Appearance (form)	Dry powder	Visual	Powder or agglomerates	Spray-dried powder
Appearance in solution	Clear, colorless to slightly yellow	Visual	NS	NS
Identity (6'-SL sodium salt)	Conform to reference standard, 6'-SL sodium salt derived from human milk	HPAEC-PAD	RT of standard ±3%	NS
pH	4 to 7 (20°C, 10% solution)	Eurofins' internal method, potentiometry	4.5 to 6.0 (20°C, 5% solution)	NS
Carbohydrates, %DM				
6'-SL sodium salt	NLT 88.0	HPAEC-PAD	NLT 90.0 w/w %	NLT 90.0 w/w %
Lactose	NMT 5.0	HPAEC-PAD	NMT 5.0 w/w %	NMT 5 w/w %
Sialic acid	NMT 5.0	HPAEC-PAD	NMT 2.0 w/w %	NMT 10 w/w %
Sum of other carbohydrates[a,b]	NMT 10.0	HPAEC-PAD	NMT 3.0 w/w %	NMT 10 w/w %
Sodium, Na	NMT 45 g/kg	ICP-AES	2.5 to 4.5 w/w %	NMT 4.2%
Chemical Analysis				
Water content	NMT 7.0 w/w %	Karl-Fischer, volumetric	NMT 6.0 w/w %	NMT 9.0 w/w %
Protein content	NMT 100 µg/g	Roti®-Nanoquant	NMT 0.01 w/w % (by Bradford assay)	NMT 100 µg/g
Ash content[c]	NMT 8.5%	NEN 6810 (500-550 °C)	NS	NMT 8.5%
Endotoxins (IU = EU)	NMT 300 IU/g	Ph. Eur. 2.6.14	NMT 10,000 EU/g	NMT 10,000 EU/g

Table 2.3.1-1 Product Specifications for Inbiose's 6'-SL Sodium Salt in Comparison to the 6'-SL Ingredient in GRN 881 and 922 (U.S. FDA, 2020a, 2021)

Parameter	Specification for Inbiose's 6'-SL	Method of Analysis Employed by Inbiose	Glycom's 6'-SL Sodium Salt (GRN 881) (U.S. FDA, 2020a)	Jennewein Biotechnologie's 6'-SL Sodium Salt (GRN 922) (U.S. FDA, 2021)
Heavy Metals				
Arsenic	NMT 0.05 mg/kg	ICP-MS	NS	NMT 0.2 mg/kg
Cadmium	NMT 0.01 mg/kg	ICP-MS	NS	NMT 0.1 mg/kg
Lead	NMT 0.05 mg/kg	ICP-MS	NMT 0.1 mg/kg	NMT 0.02 mg/kg
Mercury	NMT 0.01 mg/kg	ICP-MS	NS	NMT 0.5 mg/kg
Microbiological Contaminants				
Standard Plate Count	NMT 10,000 CFU/g	ISO 4833	NMT 1,000 CFU/g	NMT 10,000 CFU/g
Yeasts[d]	NMT 100 CFU/g	ISO 7954	NMT 100 CFU/g	NMT 100 CFU/g
Molds[d]	NMT 100 CFU/g	ISO 7954	NMT 100 CFU/g	NMT 100 CFU/g
Coliform	NMT 10 CFU/g	ISO 4832	NS	NMT 10 CFU/g
Enterobacteriaceae	Absent in 10 g	ISO 21528-1	NMT 10 CFU/g	NMT 10 CFU/g
Salmonella spp.	Absent in 25 g	ISO 6579-1	Absent in 25 g	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	ISO/TS 22964	NS	Absent in 10 g
Listeria monocytogenes	Absent in 25 g	AFNOR EGS 38/05-03/17	NS	NS
Bacillus cereus	NMT 10 CFU/g	ISO 7932	NS	NS
Additional				
Chloride by IC	NMT 0.10%	Discrete Analyzer, derived from ISO 2918	NMT 1.0 w/w %	NS

6'-SL = 6'-sialyllactose; CFU = colony forming units; EU = endotoxin unit; Glycom = Glycom A/S; GRN = GRAS Notice; HPAEC-PAD = high-performance anion exchange chromatography coupled with pulse amperometric detection; IC = ion chromatography; ICP-AES = inductively coupled plasma atomic emission; ICP-MS = inductively coupled plasma mass spectrometry; ISO = International Organization for Standardization; IU = international units; Jennewein = Jennewein Biotechnologie GmbH; NLT = not less than; NMT = not more than; NS = not specified; Ph. Eur. = European Pharmacopoeia; RT = retention time.

[a] Expressed in area %.

[b] Including sialic acid, lactose, and other carbohydrates.

[c] Major constituents of the ash are sodium and its (oxidized) derivatives, as well as sulfates and phosphates.

[d] Specification for yeast and mold is combined.

2.3.2 Batch Analysis

Results for the analyses of 6 non-consecutive batches of 6'-SL produced by 2 different strains are summarized in Table 2.3.2-1. The data demonstrate that the production process as described in Section 2.2 results in a consistent product that meets the established product specifications.

Table 2.3.2-1 Analytical Data Obtained from 6 Batches of 6'-SL

Parameter	Specification	First-generation Production Strain (INB_6SL_01)			Second-generation Production Strain (INB_6SL_02)		
		Lot Nos.			Lot Nos.		
		ilex09F14	ilex09F15	ilex09F17	ilex16F01	Ilex16F02	Ilex16F03
Identification							
Appearance (color)	White	White	White	White	White	White	White
Appearance (form)	Dry powder	Dry powder	Dry powder	Dry powder	Dry powder	Dry powder	Dry powder
Appearance in solution	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow
pH (20°C, 10% solution)	4 to 7	5.03	6.25	4.82	6.34	5.40	5.15
Carbohydrates, water free (%DM)							
6'-SL sodium salt	≥88	89.35	93.94	95.25	93.96	88.04	95.20
D-Lactose	≤5	0.71	0.81	0.59	2.57	3.14	0.39
Sialic acid	≤5	0.33	0.25	0.33	0.26	0.79	0.18
Other carbohydrates[a,b]	≤10	4.88	3.60	2.68	4.68	6.45	1.16
Chemical Analysis							
Water content, volumetric (w/w %)	≤7.0	6.2	6.6	4.3	5.3	4.8	3.6
Protein content (µg/g)	≤100	<25	31	<25	34	35	<25
Ash content (%) [c]	≤8.5	7.67	7.56	7.49	7.35	7.25	7.69
Sodium, Na (g/kg)	≤45	33.1	33.9	33.9	31.4	32.1	33.6
Endotoxins (IU/g)	≤300	215	<50	<50	75	<50	<50
Heavy Metals							
Arsenic (mg/kg)	≤0.02	<0.02	<0.01	<0.01	<0.01	<0.01	<0.01
Cadmium (mg/kg)	≤0.01	<0.01	<0.005	<0.005	<0.005	<0.005	<0.005
Lead (mg/kg)	≤0.05	<0.02	0.024	<0.01	<0.01	<0.01	0.02
Mercury (mg/kg)	≤0.01	0.0017	0.00053	<0.0005	<0.01	<0.01	<0.01
Microbiological Contaminants							
Standard plate count (CFU/g)	≤10,000	760	<10	<10	140	<100	<100
Yeasts[d] (CFU/g)	≤100	<10	<10	<10	<10	<10	<10
Molds[d] (CFU/g)	≤100	<10	<10	<10	<10	<10	<10
Coliforms	<10	<10	<10	<10	<10	<10	<10

Table 2.3.2-1 Analytical Data Obtained from 6 Batches of 6'-SL

Parameter	Specification	First-generation Production Strain (INB_6SL_01)			Second-generation Production Strain (INB_6SL_02)		
		Lot Nos.			Lot Nos.		
		ilex09F14	ilex09F15	ilex09F17	ilex16F01	Ilex16F02	Ilex16F03
Enterobacteriaceae	Absent in 10 g	Absent	Absent	Absent	Absent	Absent	Absent
Salmonella	Absent in 25 g	Absent	Absent	Absent	Absent	Absent	Absent
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Absent	Absent	Absent	Absent	Absent	Absent
Listeria monocytogenes	Absent in 25 g	Absent	Absent	Absent	Absent	Absent	Absent
Bacillus cereus (CFU/g)	≤10	<10	<10	<10	<10	<10	<10

6'-SL = 6'-sialyllactose; CFU = colony forming units; DM = dry matter; IU = International units.

[a] Expressed in area %.

[b] Including sialic acid, lactose, sialyllactulose, and other carbohydrates.

[c] Major constituents of the ash are sodium and its (oxidized) derivatives, as well as sulfates and phosphates.

[d] Specification for yeast and mold is combined.

2.3.3 Microbiological Endotoxins and Residual Protein Analysis

The content of endotoxins and residual proteins in the 6'-SL product is determined by methods with high sensitivity [Protein content: Roti®Nanoquant method, based on the Bradford assay; and Endotoxins: kinetic-chromogenic test (Method D) described in the European Pharmacopoeia], to ensure the consistency and quality of the 6'-SL product. Specifications for microbial endotoxin and protein are equivalent to limits established for other HiMOs that have been concluded to be GRAS (*e.g*., GRN 881) (U.S. FDA, 2020a).

2.3.4 Residual DNA Analysis

To ensure the absence of residual DNA of the production organism, PCRs were performed on the 6'-SL product of 6 regulatory batches: 3 regulatory batches with INB-6SL_01 and 3 regulatory batches with INB-6SL_02. The protocol followed the European Food Safety Authority (EFSA) guidelines for the presence of recombinant DNA. A short subsequence of the inserted *N*-acylglucosamine 2-epimerase gene of *Bacteroides ovatus* on the genome and a subsequence of the *N*-acylneuraminate cytidylyltransferase gene of *P. multocida* on the plasmid pINB-6SL_01 were targeted to check for residual DNA in the 6'-SL product from batches with INB-6SL_01 and INB-6SL_02. For every batch, the analysis was performed in triplicate together with 3 types of positive controls and 1 negative control. The analysis of all regulatory batches of 6'-SL showed no detectable levels of residual DNA in the final product. The limit of detection for the *N*-acylglucosamine 2-epimerase gene subsequence and the *N*-acylneuraminate cytidylyltransferase gene subsequence were both below the threshold limit of detection of 10 ng DNA per gram 6'-SL as it is stated in the EFSA guidelines (EFSA, 2018).

2.4 Stability

The stability of Inbiose's 6'-SL sodium salt is supported by the real-time and accelerated stability studies summarized in GRN 881 (U.S. FDA, 2020a). The compositional similarities between Inbiose's 6'-SL sodium salt and the 6'-SL sodium salt ingredients summarized in GRN 881 and 922 (see Section 2.3.1), indicate that stability of the ingredients will be similar. A summary of the real-time and accelerated stability studies, as described in GRN 881 and 922 is provided below (U.S. FDA, 2020a, 2021).

As described in Section 2.4 of GRN 881, the chemical, physical, microbiological, and sensory testing stability of Glycom's 6'-SL sodium salt (produced from fermentation) was assessed in an ongoing 5-year study under real-time conditions [25°C, 60% relative humidity (RH)] (U.S. FDA, 2020a). The 12-month interim results of 2 representative batches confirmed that, when stored at ambient room temperatures, 6'-SL sodium salt is stable. The results of an accelerated stability study (40°C, 75% RH) also indicated no changes in the evaluated physical (appearance and color), chemical (6'-SL, lactose, sialic acid, largest unspecified impurity, unspecified impurities, and water content) and microbiological parameters [aerobic mesophilic total plate count, Enterobacteriaceae, *Salmonella* spp., *Cronobacter (Enterobacter) sakazakii*, *Listeria monocytogenes*, *Bacillus cereus*, yeasts, and molds] in 2 representative batches following storage for up to 12 months. The Arrhenius equation (Peleg *et al*., 2012) was used to extrapolate the results of the accelerated stability study to conclude that the ingredient was stable for at least 5 years when protected from light and stored at room temperature and ambient humidity.

Further to this, Glycom's 6'-SL sodium salt was subject to stress/forced stability conditions. In the solid-state testing, 6'-SL sodium salt (in powdered form) was stored for 28 days at 80°C at ambient and high humidity. A "*negligible concurrent increase of lactose and sialic acid*" and "*slight isomerization of 6'-SL to 6'-sialyl-lactulose*" were observed, increasing with humidity conditions. In the aqueous solution testing, 6'-SL sodium salt was exposed to a pH range (3.0 to 9.0) for 28 days at 35°C or an acid (0.1 N HCl) and base (0.01 N NaOH) for 24 hours at 35°C. The 6'-SL sodium salt was stable at neutral pH (6.7 and 6.9) and a minor hydrolysis of 6'-SL sodium salt to sialic acid and lactose was observed at a slightly acidic pH (5.0). The 6'-SL sodium salt was found to be almost completely hydrolyzed to sialic acid and lactose under the acidic conditions (pH 3.0, 35°C for 1 month or at 0.1 N HCl, 35°C for 24 hours). A significant (10 to 30%) isomerization of 6'-SL sodium salt to 6'-sialyl-lactulose was noted under the basic conditions (pH 9.0, 35°C for 1 month or at 0.01 N NaOH 35°C for 24 hours).

The stability of Jennewein's 6'-SL sodium salt was assessed in a HMO mixture containing approximately 4% 6'-SL, stored in high-density polyethylene bottles under ambient (25°C, 60% RH) and accelerated (40°C, 75% RH) conditions for 52 and 26 weeks, respectively (Part II, Section H; GRN 922) (U.S. FDA, 2021). Under ambient conditions, 6'-SL content remained relatively unchanged and moisture increased from 5.7 to 7.8%. Under accelerated conditions, the 6'-SL content decreased and moisture content increased. These data supported the 1-year shelf life of 6'-SL sodium salt when stored under ambient conditions.

The stability of 6'-SL sodium salt was also assessed under the intended conditions of use. The 6'-SL sodium salt ingredient produced by Glycom was assessed in a powdered infant formula supplemented with other HiMOs, long chain polyunsaturated fatty acids (LC-PUFA), vitamins, and minerals, as described in Section 2.4.2 of GRN 881 (U.S. FDA, 2020a). The 6'-SL sodium salt in infant formula was found to be stable up to 12 months of storage at various temperatures (4°C, 20°C, 30°C, and 37°C). As a constitutional isomer, the stability testing of GeneChem's 3'-SL in milk and yogurt was used to support the stability of 6'-SL sodium salt in milk (content, appearance, and odor) for 45 days at 4±2°C and 25±2°C and within the targeted stability range for 45 days in low temperature yogurt (4±2°C, 26±3% RH). The 3'-SL content did not comply with target stability ranges after 15 days in room temperature yogurt (25±2°C, 25±6% RH) (Section 2.C.5.2, GRN 766) (U.S. FDA, 2018b).

These results indicate that 6'-SL sodium salt is anticipated to be stable in most food matrices.

Part 3. § 170.235 Dietary Exposure

3.1 Estimated Intake of 6'-SL

3.1.1 Methods

Inbiose's 6'-SL sodium salt is intended to be added to non-exempt term infant formula, foods targeted to infants and young children and specific used in food and beverage products for the general U.S. population (see Table 1.3-1). Proposed food uses and use levels are hereby incorporated by reference to Part 3 § 170.235 Dietary Exposure of GRN 881 (U.S. FDA, 2020a). As food uses of 6'-SL sodium salt are fully substitutional to current GRAS uses previously determined to be GRAS in GRN 881, no change in dietary intake of 6'-SL sodium salt are expected from the introduction of Inbiose's 6'-SL sodium salt ingredient to the U.S. marketplace (U.S. FDA, 2020a). A summary of the estimated dietary intake of 6'-SL sodium salt from food uses described in GRN 881 are presented below and are considered applicable to GRAS uses of 6'-SL sodium salt described herein (U.S. FDA, 2020a).

Jennewein's GRN 922 is based on the same concentration as what has been concluded GRAS in GRN 881 (U.S. FDA, 2020a, 2021).

3.1.2 Intake Estimates for 6'-SL Sodium Salt

As described in Part 3.2 of GRN 881, the estimated intake of 6'-SL sodium salt as an ingredient in term infant formula (0 to 12 months), toddler formula and other food and beverage products has been estimated from dietary survey data (U.S. FDA, 2020a). The intake of 6'-SL sodium salt described in GRN 881 was estimated using food categories representative of each proposed food use chosen from the National Center for Health Statistics' 2013-2014 National Health and Nutrition Examination Survey (NHANES) (CDC, 2015, 2016; USDA, 2016). Based on the proposed uses, more than 80.1% of the evaluated population groups consisted of eligible 6'-SL sodium salt consumers, with infants (7 to <12 months) at 99.9% representing the greatest proportion of potential consumers. They were also established to represent the highest mean and 90th percentile consumer-only intakes of 6'-SL sodium salt on an absolute basis, at 0.88 and 1.64 g/person/day, respectively. The summary of the estimated dietary intake of 6'-SL sodium salt in the U.S. population, as described in GRN 881, is provided in Table 3.1.2-1 (U.S. FDA, 2020a).

Table 3.1.2-1 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)[b]

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; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; 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] Table adapted from GRN 881 (U.S. FDA, 2020a), full intake assessment reported in GRN 881 GRAS evaluation.

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

The first GRAS conclusion notified to the U.S. FDA for 6'-SL sodium salt was submitted by Glycom in 2019 (GRN 881; U.S. FDA, 2020a). A critical and comprehensive review of the publicly available data and information pertaining to the safety of 6'-SL sodium salt for use as an ingredient in non-exempt infant formula, and various food and beverage products across multiple categories was presented in the Notice, and the published information pertinent to safety of 6'-SL sodium salt presented by Glycom has served as the basis for a subsequent GRAS evaluation for a similar 6'-SL sodium salt preparation (U.S. FDA, 2021). These 6'-SL sodium salt preparations are produced *via* microbial fermentation using genetically modified strains of *E. coli* K-12 DH1 or *E. coli* BL21 DE3. Despite differences in manufacturing process, these 6'-SL sodium salt ingredients are all compositionally highly similar (see Table 2.3.1-1) and therefore safety data conducted with any of these ingredients are generally applicable to all ingredients. Within the previous GRAS Notices, data and information supporting the GRAS use of 6'-SL sodium salt as an ingredient in infant formula and other foods have been critically reviewed by a number of qualified scientific experts, including the FDA, and are publicly available. Additionally, EFSA has recently issued an opinion supporting the safe use of 6'-SL sodium salt as an ingredient in a variety of foods, including infant and follow-on formula (EFSA, 2020).

As reported in Part III, Section B of GRN 922, reported concentrations of 6'-SL in human milk can range from 0.1 to 0.8 g/L and unlike most other HMOs, concentrations of 6'-SL were not found to differ between Secretor status of the mothers (U.S. FDA, 2021). However, variations in concentrations were reported in geographical location and lactation time (Austin *et al*., 2016; Kunz *et al*., 2017; McGuire *et al*., 2017; Sprenger *et al*., 2017; Thurl *et al*., 2017; Azad *et al*., 2018; Nijman *et al*., 2018; Wejryd *et al*., 2018; Austin *et al*., 2019; Larsson *et al*., 2019; McJarrow *et al.*, 2019; Paganini *et al*., 2019; Samuel *et al*., 2019; Tonon *et al.*, 2019). As such, the use of 6'-SL sodium salt as an ingredient in non-exempt term infant formula at levels up to 0.4 g/L is within the range that infants are exposed to following the ingestion of human milk.

Based on the equivalence of Inbiose's 6'-SL sodium salt to other 6'-SL sodium salt preparations with GRAS status, publicly available data and information establishing the GRAS status of 6'-SL sodium salt are therefore incorporated by reference to previous GRAS evaluations in the Sections below (U.S. FDA, 2020a, 2021). Since the most recent GRAS conclusion notified to the U.S. FDA was in 2020, an updated comprehensive search of the publicly available scientific literature was conducted to identify new information relevant to the safety of 6'-SL sodium salt published through 24 March 2021. The following databases were accessed: AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine, BIOSIS Toxicology, BIOSIS Previews, CAB ABSTRACTS, Embase, Foodline: SCIENCE, FSTA, MEDLINE, NTIS: National Technical Information Service, Toxicology Abstracts, and ToxFile. A summary of the historical basis for the GRAS conclusion of 6'-SL sodium salt and any newly identified studies relevant to the safety of Inbiose's 6'-SL sodium salt are provided below.

6.2 Absorption, Distribution, Metabolism and Excretion

As discussed previously, 6'-SL produced by microbial fermentation is structurally identical to the 6'-SL found in human milk and will be physiologically equivalent, in terms of absorption, distribution, metabolism, and excretion. Therefore, the metabolism of this HiMO, when added to infant formula, is expected to be identical to those of other HMOs in human breast milk.

The metabolism of HMOs, including 6'-SL, has been previously described in detail in GRN 881 and 922 (U.S. FDA, 2020a, 2021). Briefly, HMOs are resistant to enzymatic hydrolysis and are therefore not significantly digested in the upper gastrointestinal tract (Brand-Miller *et al*., 1998; Engfer *et al*., 2000; Rudloff and Kunz, 2012; EFSA, 2020). Once in the large intestine, 6'-SL is metabolized by the intestinal microbiota. In an *in vitro* study of various sialyllactoses treated with artificial gastric fluid, Gnoth *et al.* (2000) observed only minor structural changes in the HMOs and concluded that <5% of those ingested would be digested and subsequently absorbed. In breastfed infants, only minimal levels of ingested HMOs have been detected unchanged in the urine (*i.e.*, 1 to 2% of the total HMO fraction).

6.3 Toxicological Studies

6.3.1 Toxicology Studies Conducted with Inbiose's 6'-SL Sodium Salt

Toxicology studies characterizing the genotoxicity and subchronic toxicity of 6'-SL sodium salt in neonatal rats is presented as conducted information on the safety of the ingredient. Findings from these studies are consistent with observations previously reported in the published literature and described in GRN 881 and 922 (U.S. FDA, 2020a, 2021): 6'-SL sodium salt is not genotoxic and does not pose a toxicological safety concern.

6.3.1.1 Genotoxicity

***In vitro* Bacterial Reverse Mutation (Ames) Test (OECD Test Guideline 471) (OECD, 1997)**

The potential mutagenicity of 6'-SL sodium salt was evaluated in a bacterial reverse mutation assay conducted in accordance with OECD Test Guideline 471 (OECD, 1997) (Chevallier, 2020a [unpublished]). The study included a Preliminary Range Finding Test (Plate Incorporation Method), an Assay 1 (Plate Incorporation Method) and an Assay 2 (Plate incorporation method without metabolic activation and Pre-Incubation Method with metabolic activation). Formulations were analyzed for concentration.

For the range finding test, *Salmonella* Typhimurium (*S.* Typhimurium) TA98 and TA100 and *E. coli* strain WP2 *uvrA* tester strains were exposed to 6'-SL sodium salt at 20.58, 61.73, 185.2, 555.6, 1,666.7, and 5,000 µg/plate in the absence and presence of metabolic activation (S9 mix prepared from an Aroclor 1254-induced rat liver post-mitochondrial fraction rats). For the plate incorporation and pre-incubation method *S.* Typhimurium strains TA98 (pKM101), TA100 (pKM101), TA1535, and TA1537 and *E. coli* strain WP2 *uvrA* were exposed to 6'-SL sodium salt at concentrations of 312.5, 625, 1,250, 2,500, and 5,000 µg/plate (the OECD Test Guideline 471 maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix) (OECD, 1997). Water for injection served as the vehicle for 6'-SL sodium salt and as the negative control. Strain-specific positive controls were also included in the presence [2-anthramine (2AM) for *S.* Typhimurium strains TA 1535, TA 1537, and TA 98, *E. coli* strain WP2 uvrA, and benzo(a)pyrene (BaP) for *S.* Typhimurium strain TA 100] and absence [sodium azide (NaN_3) for *S.* Typhimurium strains TA 1535 and TA 100, 9-Aminoacridine (9AA) for *S.* Typhimurium strain TA 1537, 2-Nitrofluorene (2NF) for *S.* Typhimurium strain TA 98 and 4-Nitroquinoline 1-oxide (4NQO) for *E. coli* strain WP2 uvrA] of metabolic activation. The full list of controls is provided below in Table 6.3.1.1-1.

There was no evidence of mutagenicity in either test, in the absence or presence of metabolic activation. The mean number of revertant colonies did not show any biologically relevant increase compared to the solvent controls. There were no reproducible dose-related trends and there was no indication of any treatment-related effect. No precipitation nor growth inhibition and no cytotoxic effects of 6'-SL sodium salt were observed in the main assays.

The mean values of revertant colonies of the negative (vehicle/solvent) control plates were within the historical control range. The positive controls showed a distinct increase of induced revertant colonies in each strain with and without metabolic activation. The study was considered to be valid.

Under the experimental conditions applied in this study, 6'-SL sodium salt did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. The results of Inbiose's bacterial reverse mutation assay are provided below in Table 6.3.1.1-2.

Based on the results of the study, it was concluded that 6'-SL sodium salt is non-mutagenic at concentrations up to 5,000 µg/plate (the OECD Test Guideline 471 maximum recommended concentration) (OECD, 1997).

Table 6.3.1.1-1 List of Controls Used for Inbiose's *In vitro* Bacterial Reverse Mutation Assay

Without Metabolic Activation (-S9 mix)					
Test Strains	**TA 1535**	**TA 1537**	**TA 98**	**TA 100**	**WP2 uvrA**
Positive control	NAN_3, 1 µg/plate	9AA, 50 µg/plate	2NF, 0.5 µg/plate	NAN_3, 1 µg/plate	4NQO, 2 µg/plate
With Metabolic Activation (+S9 mix)					
Test Strains	**TA 1535**	**TA 1537**	**TA 98**	**TA 100**	**WP2 uvrA**
Positive control	2AM, 2 µg/plate	2AM, 2 µg/plate	2AM, 2 µg/plate	BaP 5 µg/plate	2AM, 10 µg/plate

2AM = 2-anthramine; 2NF = 2-nitrofluorene; 4NQO = 4-nitroquinoline 1-oxide; 9AA = 9-aminoacridine; BaP = benzo[a]pyrene; NAN_3 = sodium azide; S9 = metabolic activation.

Table 6.3.1.1-2 Results of Inbiose's *In vitro* Bacterial Reverse Mutation Assay

Concentration (µg/plate)	Revertant Colonies per Plate (Mean ± SD)									
	Without Metabolic Activation (-S9 mix)					With Metabolic Activation (+S9 mix)				
	Salmonella Typhimurium				*Escherichia coli*	*Salmonella* Typhimurium				*Escherichia coli*
	TA1535	TA1537	TA98	TA100	WP2unA	TA1535	TA1537	TA98	TA100	WP2unA
Direct Plate Incorporation Assay										
Water for injection	19.0±1.7	6.7±3.2	14.7±2.9	111.7±16.2	18.7±1.5	18.7±2.9	6.7±2.3	20.7±0.6	138.0±12.1	32.3±6.4
5,000	21.0 ±3.6	7.0±4.0	15.0±1.7	103.7±9.3	19.0±7.9	21.0±3.6	9.0±1.7	19.0±2.6	142.7±5.0	26.7±5.9
2,500	22.7±4.5	8.0±2.0	14.7±5.9	125.3±4.0	21.3±1.5	24.0±3.5	8.0±3.6	18.7±5.9	147.0±9.6	24.7±5.1
1,250	19.0±3.6	2.7±1.5	18.0 ±1.7	125.3±18.6	26.7±4.7	20.3±3.1	7.7±4.0	23.7±5.9	148.3±19.7	35.7±9.5
625	22.0 ±1.0	6.0±2.6	17.0±3.6	114.0±8.5	21.3±1.5	20.0±11.1	5.7±2.3	21.0±4.6	127.3±6.8	29.3±2.1
312.5	18.3±5.0	7.0±3.6	19.7±2.9	115.3±11.0	16.3±3.1	24.7±4.5	8.7±0.6	21.7±5.0	142.7±18.4	29.7±5.0
Positive control[a]	535.7±17.7	73.3±26.1	95.7±7.0	633.3±15.3	688.7±40.1	136.0±13.7	76.7±7.1	1,093.3±61.9	806.7±27.4	152.0±10.1
Direct Plate Incorporation (without S9 mix) and Pre-incubation (with S9 mix) Assay										
Water for injection	8.0±1.7	4.0±2.0	17.3±4.7	157.7±7.0	23.0±7.0	10.3± 2.3	7.3± 4.7	22.3± 8.5	172.3±15.0	35.7± 4.2
5,000	6.7±3.1	5.7±1.5	14.7± 4.5	147.0± 5.3	25.3± 3.1	10.3± 6.0	7.3±0.6	22.0±2.6	181.7±19.4	32.7±4.7
2,500	6.3±2.1	3.3±1.2	18.7± 1.5	152.3± 5.8	26.7± 10.4	9.0± 1.0	6.0±2.6	23.3±10.7	160.3±28.7	31.7±10.2
1,250	6.3±2.5	5.0±1.7	14.7± 3.5	136.3± 5.5	23.0± 5.3	10.0± 1.0	9.7±0.6	19.3±3.1	155.0±12.1	36.0±5.6
625	8.0±2.6	7. 0±1.0	18.3± 2.9	137.0±11.5	28.0± 9.5	7.0± 4.0	13.7±4.6	21.0±5.6	179.7±12.2	28.7±7.4
312.5	9.7±2.1	7.0±3.0	14.7± 2.9	121.7± 5.0	18.0± 2.0	11.7± 4.2	7.3±1.5	18.3±2.1	167.0±7.8	30.0±5.6
Positive control[a]	503.7±27.6	39.±17.7	85.7±10.3	502.3±40.0	971.3±66.5	112.0±6.6	59.7±14.6	1189.3±38.2	921.7±63.6	145.3±26.4

S9 = metabolic activation; SD = standard deviation.

[a] List of positive controls is included in Table 6.3.1.1-1.

***In vitro* Mammalian Cell Micronucleus Test (OECD Test Guideline 487) (OECD, 2016a)**

The potential clastogenicity and aneugenicity of 6'-SL sodium salt was evaluated in an *in vitro* micronucleus test with human peripheral blood lymphocytes. This study was conducted in accordance with OECD Test Guideline 487 (OECD, 2016a) (Chevallier, 2020b, unpublished]).

For the pulse exposure, the human lymphocytes were exposed to 6'-SL sodium salt at concentrations of 0 (water for injection and vehicle), 125, 250, 500, 1,000, or 2,000 6'-SL sodium salt µg/mL in the presence [Cyclophosphamide (CPA) dissolved in water at concertation of 6 µg/mL] and absence [Colchicine (Colch) dissolved in water at concertation of 0.1 µg/mL] of external metabolic activation (S9 mix) for 3 hours followed by a 24-hour recovery. For the continuous exposure, the human lymphocytes were exposed to 6'-SL sodium salt at concentrations of 0 (water for injection and vehicle), 125, 250, 500, 1,000, or 2,000 6'-SL sodium salt µg/mL in the absence [Mitomycin C (MMC) dissolved in water at concertation of 0.1 µg/mL] of external metabolic activation (S9 mix) for 24 hours with no recovery.

No precipitation of the test item was observed at the end of treatment. When compared to the vehicle control group, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells was observed in either independent experiment after treatment with the test item. The positive control cultures showed statistically significant increases in the frequency of micronucleated binucleated cells (MNBC). It was concluded that the metabolic activation system functioned properly and the study was valid. The results of Inbiose's *in vitro* mammalian cell micronucleus test are provided below in Table 6.3.1.1-3.

Based on the results of this study, 6'-SL sodium salt was concluded to have no potential for clastogenicity and aneugenicity in human lymphocytes at doses up to 2,000 µg/mL (the OECD Test Guideline 487 maximum recommended concentration) (OECD, 2016a).

Table 6.3.1.1-3 Results of Inbiose's *In Vitro* Mammalian Cell Micronucleus Test

Concentration (µg/mL)	RI as Mean % Control	Culture	Number of Analyzed Micronucleated Binucleated Cells	Total Micronucleated Binucleated Cells		Frequency of Micronucleated Binucleated Cells (%)
				Per Culture	Per Dose	
3-h Treatment: Without Metabolic Activation (-S9 mix)						
Vehicle	NA	C1	1,000	2	4	2.0
		C2	1,000	2		
500	98	C1	1,000	5	6	3.0
		C2	1,000	1		
1,000	104	C1	1,000	3	6	3.0
		C2	1,000	3		
2,000	101	C1	1,000	1	9	4.5
		C2	1,000	8		
Colchicine (COL): 0.1 µg/mL	50	C1	660	10	26	15.0 ***
		C2	1,074	16		
3-h Treatment: With Metabolic Activation (+S9 mix)						
Vehicle	NA	C1	1,000	2	3	1.5
		C2	1,000	1		
500	103	C1	1,000	3	5	2.5
		C2	1,000	2		
1,000	96	C1	1,000	1	2	1.0
		C2	1,000	1		
2,000	97	C1	1,000	1	1	0.5
		C2	1,000	0		
Cyclophosphamide (CPA): 6 g/mL	53	C1	1,000	37	64	32.0***
		C2	1,000	27		

Table 6.3.1.1-3 Results of Inbiose's *In Vitro* Mammalian Cell Micronucleus Test

Concentration (µg/mL)	RI as Mean % Control	Culture	Number of Analyzed Micronucleated Binucleated Cells	Total Micronucleated Binucleated Cells		Frequency of Micronucleated Binucleated Cells (%)
				Per Culture	Per Dose	
24-h Treatment: Without Metabolic Activation (-S9 mix)						
Vehicle	NA	C1	1,000	2	5	2.5
		C2	1,000	3		
500	111	C1	1,000	2	6	3.0
		C2	1,000	4		
1,000	107	C1	1,000	4	4	2.0
		C2	1,000	0		
2,000	104	C1	1,000	4	6	3.0
		C2	1,000	2		
Mitomycin C (MMC): 0.1 µg/mL	77	C1	1,000	49	93	46.5***
		C2	1,000	44		

C1 = culture 1; C2 = culture 2; h = hour(s); NA = not applicable; RI = replication index.
Vehicle: water for injection.

***In vitro* Mammalian Chromosomal Aberration Test (OECD Test Guideline 473) (OECD, 2016b)**

The potential for 6'-SL to cause structural chromosomal aberrations in an *in vitro* mammalian chromosomal aberration test with human peripheral blood lymphocytes. This study was conducted in accordance with OECD Test Guideline 473 (OECD, 2016b) (Chevallier, 2020c [unpublished]).

For the pulse exposure, the human lymphocytes were exposed to 6'-SL sodium salt at concentrations of 0 (water for injection and vehicle), 125, 250, 500, 1,000, or 2,000 6'-SL sodium salt µg/mL for 3 hours followed by a 20-hour recovery in the presence (CPA dissolved in water at concertation of 12.5 and 25 µg/mL) and absence (MMC dissolved in water at concertation of 2 and 3 µg/mL) of external metabolic activation (S9 mix). For the continuous exposure, the human lymphocytes were exposed to 6'-SL sodium salt at concentrations of 0 (water for injection and vehicle), 125, 250, 500, 1,000, or 2,000 6'-SL sodium salt µg/mL for 20 hours with no recovery in the absence (MMC dissolved in water at concertation of 0.2 and 0.3 µg/mL) of external metabolic activation (S9 mix).

None of the test concentrations exhibited a statistically significant increase in the frequency of cells with structural chromosomal aberrations when compared to the negative control, and the results were within historical negative control data. There was a statistically significant increase in the frequencies of cells with structural chromosome aberrations for positive controls over the background frequency of the vehicle control cultures. Hence, the acceptance criteria were met. The study was considered to be valid. The results of each experiment are provided below in Tables 6.3.1.1-4 to 6.3.1.1-6.

Based on the results of this study, 6'-SL sodium salt was concluded to have no potential for induction of chromosomal aberrations in human lymphocytes at doses up to 2,000 µg/mL (the OECD Test Guideline 473 maximum recommended concentration) (OECD, 2016b).

Table 6.3.1.1-4 Main Experiment without S9 Mix: Chromosome Aberration (3-Hour Treatment, 20-Hour Recovery)

Doses µg/mL	Slide Nb.	Nb. of cells scored	NA	Structural chromosome aberrations (type and number)									Cells with structural chromosome aberrations			
				G	Chromatid		Chromosome		MA	PU	Total +G	Total -G	Nb. +G	Frequency (%) +G	Nb. -G	Frequency (%) -G
					D	Exch	D	Exch								
0	32 C1	150	0	1	4	0	0	0	0	0	**8**	7	5	**2.7**	4	**2.3**
	48 C2	150	0	0	2	1	0	0	0	0			3		3	
500	54 C1	150	1	1	0	0	0	0	0	0	**2**	**0**	1	**0.7**	0	**0.0**
	69 C2	150	0	1	0	0	0	0	0	0			1		0	
1000	28 C1	150	0	0	0	0	0	0	0	0	**1**	**0**	0	**0.3**	0	**0.0**
	44 C2	150	0	1	0	0	0	0	0	0			1		0	
2000	68 C1	150	0	0	0	1	0	0	0	0	**4**	**4**	1	**1.3**	1	**1.3**
	33 C2	150	0	0	3	0	0	0	0	0			3		3	
MMC	63 C1	50	1	1	14	12	1	1	2	0	**64**	**59**	23	**43.0**	23	**42.0**
2 µg/mL	39 C2	50	0	4	10	15	4	0	0	0			20		19	***

Nb.: number
NA: numerical aberrations, G: gap, D: deletion, Exch: exchange, MA: multiple aberrations, PU: pulverization.
C1: culture 1 (Female)
C2: culture 2 (Female)
0: vehicle control (water for injections)
MMC: Mitomycin C
Statistical analysis: χ^2 test ***: $p < 0.001$ (performed only for cells with structural aberrations excluding gaps)

Table 6.3.1.1-5 Main Experiment with S9 Mix: Chromosome Aberration (3-Hour Treatment, 20-Hour Recovery)

Doses µg/mL	Slide Nb.	Nb. of cells scored	NA	Structural chromosome aberrations (type and number)									Cells with structural chromosome aberrations			
				G	Chromatid		Chromosome		MA	PU	Total +G	Total -G	Nb. +G	Frequency (%) +G	Nb. -G	Frequency (%) -G
					D	Exch	D	Exch								
0	42 C1	150	0	2	1	0	0	0	0	0	**5**	**2**	3	**1.7**	1	**0.7**
	26 C2	150	0	1	1	0	0	0	0	0			2		1	
500	52 C1	150	0	1	1	0	0	0	1	0	**4**	**2**	3	**1.3**	2	**0.7**
	65 C2	150	0	1	0	0	0	0	0	0			1		0	
1000	40 C1	150	0	1	0	0	0	0	0	0	**2**	**1**	1	**0.7**	0	**0.3**
	46 C2	150	0	0	1	0	0	0	0	0			1		1	
2000	55 C1	150	0	3	1	0	0	0	0	0	**5**	**2**	4	**1.7**	1	**0.7**
	67 C2	150	0	0	0	0	1	0	0	0			1		1	
CPA	30 C1	50	0	2	11	3	1	0	1	0	**40**	**38**	15	**29.0**	14	**28.0**
25 µg/mL	36 C2	50	0	0	14	5	2	0	1	0			14		14	***

Nb.: number
NA: numerical aberrations, G: gap, D: deletion, Exch: exchange, MA: multiple aberrations, PU: pulverization.
C1: culture 1 (Female)
C2: culture 2 (Female)
0: vehicle control (water for injections)
CPA: Cyclophosphamide
Statistical analysis: χ^2 test ***: $p < 0.001$ (performed only for cells with structural aberrations excluding gaps)

Table 6.3.1.1-6 Main Experiment without S9 mix: Chromosome Aberration (20-Hour Treatment, No recovery)

Doses µg/mL	Slide Nb.	Nb. of cells scored	NA	Structural chromosome aberrations (type and number)									Cells with structural chromosome aberrations			
				G	Chromatid		Chromosome		MA	PU	Total +G	Total -G	Nb. +G	Frequency (%) +G	Nb. -G	Frequency (%) -G
					D	Exch	D	Exch								
0	29 C1	150	0	0	0	0	2	0	0	0	**2**	**2**	2	**0.7**	2	**0.7**
	23 C2	150	0	0	0	0	0	0	0	0			0		0	
500	61 C1	150	1	0	2	2	0	0	0	0	**6**	**5**	4	**2.0**	4	**1.7**
	57 C2	150	1	1	1	0	0	0	0	0			2		1	
1000	37 C1	150	0	1	3	0	1	0	1	0	**9**	**7**	6	**3.0**	5	**2.3**
	25 C2	150	0	1	2	0	0	0	0	0			3		2	
2000	50 C1	150	0	2	0	0	0	0	0	0	**5**	**2**	2	**1.7**	0	**0.7**
	47 C2	150	1	1	2	0	0	0	0	0			3		2	
MMC	24 C1	50	0	0	11	4	1	0	0	0	**33**	**32**	14	**26.0**	14	**26.0**
0.2 µg/mL	56 C2	50	0	1	6	8	2	0	0	0			12		12	***

Nb.: number
NA: numerical aberrations, G: gap, D: deletion, Exch: exchange, MA: multiple aberrations, PU: pulverization.
C1: culture 1 (Female)
C2: culture 2 (Female)
0: vehicle control (water for injections)
MMC: Mitomycin C
Statistical analysis: χ^2 test ***: $p < 0.001$ (performed only for cells with structural aberrations excluding gaps)

6.3.1.2 Acute Oral Toxicity Test (OECD Test Guideline 425) (OECD, 2008)

The median lethal dose (LD_{50}) of 6'-SL sodium salt was assessed in a single dose acute toxicity study in accordance with OECD Test Guideline 425 (OECD, 2008; Tarcai, 2020 [unpublished]). Three female Crl:WI Wistar rats were administered a single dose of 5,000 mg 6'-SL salt/kg body weight dissolved in distilled water *via* gavage, followed by a 14-day observation period. No mortality nor test item-related effects were observed during the study; hence, the LD_{50} was concluded to be greater than 5,000 mg 6'-SL sodium salt/kg body weight (the OECD Test Guideline 425 maximum recommended concentration) (OECD, 2008).

6.3.1.3 Repeat Dose Toxicity Study (Dose Range Finding Study)

A non-Good Laboratory Practice (GLP) 21-day repeat dose toxicity study was conducted in juvenile Sprague-Dawley (SD) rats to evaluate the short-term toxicity of 6'-SL sodium salt and select a maximum dose for the subsequent 90-day subchronic toxicity study (Spézia, 2020 [unpublished]). Groups of 8 male and female juvenile SD rats were administered 0 (sterile water for injection, vehicle) 3,000, 4,000, or 5,000 mg 6'-SL/kg/day by gavage from Post-natal Day (PND) 7 to 27 at 10 mL/kg/day. In addition, a group of satellite SD rats (6/sex/group) was administered 6'-SL sodium salt under the same conditions as the principal animals. After the treatment, the blood and urine samples were collected and used for the method validation of 6'-SL sodium salt concentration in urine and plasma samples.

All animals were observed daily for mortality and clinical signs. Body weight and food consumption (after weaning) were recorded at designated intervals. Hematology and blood chemistry parameters were measured at the end of the treatment period. Urine samples were taken from the first 5 surviving animals/sex/group to determine test item levels and toxicokinetic assessments were conducted from plasma samples of 6 pups/sex/group on PND 27 at 0, 1, 2, 4, 6, and 24 hours. Satellite animals were sacrificed following collection of their last blood sample.

On PND 28 (after at least 14 hours of fasting), the principal animals were euthanized, and a complete macroscopic post-mortem examination of the principal thoracic and abdominal organs was performed. Selected organs were weighed.

There were 4 unscheduled deaths in in test item-treated groups with 3,000, 4,000, and 5,000 6'-SL mg/kg body weight (bw)/day. A principal male found dead on PND 18 (approximately 2 hours after dosing) had yellowish colour feces on PND 16 and 17. At necropsy, the cecum was distended with gas, the colon/jejunum/ileum had orange content; and the lungs/bronchi had diffused red discoloration. A principal female found dead on PND 20 (in the morning, before treatment) had yellowish colour feces on PND 15 and 16. At necropsy, the cecum had red discoloration and the jejunum/ileum/stomach was distended with gas. The cause of these deaths could not be clearly established. No necropsies were performed for dead satellite animals.

Abnormal yellowish feces color accompanied by soiled urogenital region was observed in most pups administered 3,000 to 5,000 mg 6'-SL/kg/day. These clinical signs, appearing from PND 14 up to PND 20 were considered to be test item-related. The yellowish feces were also noted until weaning in juvenile rats exposed by gavage to lacto-*N*-neotetraose (LNnT) and fructo-oligosaccharides (FOS) exposure (Coulet *et al*. (2013), this clinical sign was considered to not be adverse.

No effects on mean body weight, mean body weight change, mean food consumption, and on mean hematology or blood biochemistry parameters, up to 5,000 mg/kg bw/day were observed. In addition, there were no test item-related organ weight changes or gross findings. The few macroscopic findings were consistent with spontaneously occurring findings described in the literature, as the findings were distributed randomly among groups, and/or their appearance was similar to findings found in controls. At microscopic examination, there was a minimally increased infiltrate of mixed inflammatory cells in the cecum from males and females treated at all dose levels associated with minimal reactive epithelial cell proliferation. In view of the low severity and absence of deleterious associated changes, these changes were considered to be non-adverse. When compared to the controls, some statistically significant differences were observed in organ weight changes; however, these were determined to not be dose-related and hence, unlikely to be test-item-related. A summary of the statistically significant observations in the 21-day dose range finding study using Inbiose's 6'-SL sodium salt ingredient is provided below in Table 6.3.1.3-1.

As the cause of death of 2 animals could not be clearly established, 4,000 mg/kg bw/day was considered to be the acceptable high-dose level for the 90-day subchronic study.

Table 6.3.1.3-1 Summary of the Statistically Significant Observations in 21-day Dose Range Finding Study Using Inbiose's 6'-SL Sodium Salt Ingredient

Parameters	Exposure	Sex	Dose Group (6'-SL sodium salt mg/kg bw/day)			
			0	3,000	4,000	5,000
Body Weight/Mean Body Weight Change (Mean values ± SD)						
Body weight (g) (1)	Day 8	M	36±3.2	**40*±3.1**	38±2.7	39±3.1
Body Weights Change (g) (1)	Days 1/4	M	7±1.0	**8*±0.7**	8±1.0	**8*±0.9**
Body Weights Change (g) (1)	Days 8/11	F	6±1.8	**8*±1.0**	7±1.2	**8*±1.2**
Body Weights Change (g) (1)	Days 18/20	F	13±1.4	12±1.4	**11*±1.3**	12±1.1
Hematology (Mean values ± SD)						
RBC (T/L) (3)	Week 4	M	5.35±0.203	**5.90*±0.281**	5.52±0.183	5.59±0.088
MCV (fL) (3)	Week 4	M	67.5±1.30	**62.3** ±1.38**	67.2±1.78	64.6±0.98
MCH (pg) (3)	Week 4	M	20.4±0.33	**18.9*±0.39**	20.5±0.46	19.6±0.38
PLT (G/L) (3)	Week 4	M	1,095±44.6	**1,278*±64.5**	1,171±78.6	**1,299**±61.4**
RTC (%) (3)	Week 4	M	13.39±1.018	**10.39**±0.936**	11.17±0.288	11.74±0.626
RTC (T/L) (3)	Week 4	M	0.72±0.048	**0.61*±0.027**	**0.62*±0.031**	0.65±0.029
N (G/L) (3)	Week 4	M	0.21±0.061	0.50±0.271	0.23±0.006	**1.06**±0.505**
Mo (G/L) (3)	Week 4	M	0.03±0.000	0.05±0.016	0.03±0.017	**0.13*±0.080**
Blood Biochemistry (Mean values ± SD)						
Na+ (mmol/L) (1)	Week 4	M	142.3±0.86	142.3± 0.79	142.9±1.18	**140.9* ±1.01**
Na+ (mmol/L) (1)	Week 4	F	143.2±0.32	142.2±1.10	142.5±0.87	**141.5** ±1.14**
TOT.BIL (µmol/L) (1)	Week 4	M	1.68±0.264	**1.05**±0.518**	**1.09*±**0.338**	**1.12*±**0.349**
CHOL (mmol/L) (1)	Week 4	M	2.87±0.100	**2.48**±0.117**	2.69±0.265	2.62±0.289
CHOL (mmol/L) (1)	Week 4	F	2.92±0.290	**2.45**±0.214**	**2.59*±0.220**	**2.56*±0.182**
TRIG (mmol/L) (1)	Week 4	M	0.59±0.229	0.43±0.122	**0.35*±0.133**	0.41 ±0.099
PHOS (mmol/L) (1)	Week 4	F	3.26±0.302	**2.92*±0.186**	3.21±0.303	**2.88*±0.082**

6'-SL = 6'-siayllactose; CHOL = total cholesterol; F = female; M = male; Mo = monocytes; MCH = mean cell hemoglobin; MCV = mean cell volume; N = neutrophils; Na+ = sodium; PHOS = inorganic phosphorus; PLT = thrombocytes; RBC = erythrocytes; RTC = reticulocytes; SD = standard deviation; TOT.BIL = total bilirubin; TRIG = triglycerides.
* P<0.05, ** P<0.01
(1) DUNNETT TEST
(3) DUNN TEST
Assigned control group(s) : 1.

6.3.1.4 Subchronic Toxicity Study (OECD Test Guideline 408) (OECD, 2018)

A GLP subchronic toxicity study was conducted to evaluate the potential toxicity of Inbiose's 6'-SL sodium salt (Haag, 2021 [unpublished]) in which juvenile SD rats (10/sex/group) were administered 1,500, 2,500, or 4,000 mg/kg bw/day *via* gavage from PND 7 to PND 90. This study was conducted in accordance with Organisation for Economic Co-operation and Development Test Guideline 408 (OECD, 2018) and was designed to mimic a period of development corresponding to infancy through to adolescence. A reference control group of SD rats (10/sex/group) was administered 4,000 mg FOS/kg/day for comparison to the high-dose 6'-SL sodium salt group to assess any fiber-specific effects. Satellite animals (3/sex) were included in all groups to determine the level of test items in plasma and urine at the end of the treatment period. The animals were checked twice daily for mortality and at least once daily for clinical signs. Body weight and food consumption were recorded twice weekly from weaning until the end of the treatment period.

No test item-related deaths occurred during the study. Two deaths that occurred in principal animals at 4,000 mg/kg bw/day, were concluded to be technical errors during the gavage procedure, based on the macroscopic observations. Two additional deaths occurred in satellite animals; 1 control and 1 low-dose treated rat. The death of the rat from the low-dose satellite group was determined to be not test item-related.

6'-SL sodium salt was clinically well tolerated, inducing only yellowish feces in half of the animals at 2,500 or 4,000 mg/kg bw/day during Weeks 2 and 3 of the treatment period. From Week 4, yellowish feces were no longer noted. FOS also induced yellowish feces, but for a longer period (Weeks 1 to 4) and was also associated with some other adverse effects (red and soiled anus, anus wound).

No effects on pre-weaning development parameters (*e.g.*, a positive response for cliff avoidance, tooth eruption, eye opening, auditory canal opening and air righting) were noted. The long bone growth in animals administered 6'-SL sodium salt or FOS was not affected. No behavioral or neurological abnormalities attributed to 6'-SL sodium salt were observed during the tests in any animals. Also, no effects were observed on the median age at which balanopreputial separation or vaginal opening occurred in males or females given 6'-SL sodium salt. No ophthalmological findings attributed to 6'-SL sodium salt were observed during the treatment period.

No effects were noted on the hematology parameters in animals given 6'-SL sodium salt. The few statistically significant differences between control and test item-treated males (*i.e.*, lower red blood cell count at 1,500 or 4,000 mg/kg bw/day, higher mean cell volume at 1,500 mg/kg bw/day, higher reticulocyte percentage at 4,000 mg/kg bw/day and higher reticulocyte count at 2,500 mg/kg bw/day), were not attributed to the test item treatment, as they were of low magnitude and/or isolated and/or not dose-related.

No effects were noted on the blood biochemistry parameters in animals administered 6'-SL sodium salt. The few statistically significant differences between control and test item-treated males (*i.e.*, higher alkaline phosphatase activity at 1,500 mg/kg bw/day, lower potassium, glucose and total bilirubin levels at 2,500 mg/kg bw/day, lower chloride levels at 2,500 and 4,000 mg/kg bw/day) and females (lower glucose level at 1,500 mg/kg bw/day and lower sodium level at 4,000 mg/kg bw/day) were not attributed to the test item treatment as they were of low magnitude and/or not dose-related and/or occurred in 1 sex only.

The thyroid hormone levels were considered to be unaffected by treatment with the test or reference item. The statistically significant higher mean thyroid stimulating hormone (TSH) levels in females given 6'-SL

sodium salt at 2,500 or 4,000 mg/kg bw/day or FOS were not considered to be treatment-related as all individual values remained within the range of historical control data, and as these differences, which were not sex- or dose-related, did not correlate with any microscopic changes in the pituitary nor in the thyroid gland.

No effects of the test or reference item were noted on the epididymal sperm count, epididymal sperm motility or morphology, testicular sperm head count or testicular daily sperm production rate.

The non-adverse minimally increased infiltrate of mixed inflammatory cells in the cecum observed in the preliminary study were not observed in this study.

There were no test item-related macroscopic abnormalities at necropsy. All differences between 6'-SL-treated groups and controls were interpreted as incidental and/or within normal range of variation because they were present only in absolute weight or only in relative to body weight ratio, because they lacked a histological correlate, or because the difference was very small, not biologically relevant. A high incidence of kidney and urinary bladder suburothelial mononuclear cell infiltration with urothelium hyperplasia was noticed in all female groups, including vehicle controls. An ascending bacterial infection was considered as the most probable origin of the observed inflammatory changes (Frazier *et al.*, 2012). Females tend to be more susceptible, probably because the short straight urethra in the female allows easier entry of the bacteria into the bladder (Jokinen, 1990). These microscopic findings were not associated with any clinical signs or clinical pathology correlates and therefore were considered to be incidental and unrelated to the test or reference item administration since controls were affected at the same incidence and severity (minimal to marked). In addition, the inflammatory lesions in the urinary tract type were observed in other female rats obtained from the same breeder and used in concomitant studies conducted at Charles River Evreux. A summary of the statistically significant observations in 90-day juvenile rat toxicity study using Inbiose's 6'-SL sodium salt ingredient is provided below in Table 6.3.1.4-1.

It was concluded that 6'-SL sodium salt treatment did not elicit any signs of adverse toxicity.

The no-observed-adverse-effect level (NOAEL) in this study was established at 4,000 mg/kg bw/day for juvenile males and females.

Table 6.3.1.4-1 Summary of the Statistically Significant Observations in 90-day Juvenile Rat Toxicity Study using Inbiose's 6'-SL Sodium Salt Ingredient

Parameters	Exposure	Sex	Dose Group (6'-SL sodium salt mg/kg bw/day)				
			0	1,500	2,500	4,000	Reference item, FOS (4,000)
Body weights/Mean Body Weights Change (Mean values ± SD)							
Body Weights (g) (1)	Day 12	M	48±1.4	48±3.7	**43**±3.6**	47±3.5	49±2.0
Body Weights (g) (1 B)	Day 12	F	46±2.0	47± 3.9	**42*± 4.3**	47±2.7	47±1.5
Body Weights (g) (1)	Day 15	M	64±2.2	63±4.6	**58**±4.0**	62±3.6	66±1.7
Body Weights (g) (1)	Day 19	M	87 ±3.6	86 ±4.5	**80** ±4.4**	82 ±4.4	87 ±3.1
Body Weights (g) (3 K)	Day 19	F	82±1.4	82±5.8	**75** ±5.3**	81 ±2.9	82 ±1.6
Body Weights (g) (1)	Day 22	M	114±5.1	112 ±5.4	**104** ±6.3**	107** ±5.4	114 ±3.4
Body Weights (g) (3 B)	Day 22	F	104±1.9	104±7.9	**94**±6.7**	100 ±2.8	103 ±2.7
Body Weights (g) (3 K)	Day 26	M	149 ±8.2	149± 7.2	**137*±12.8**	141 ±7.7	151±4.7
Body Weights (g) (3 B)	Day 26	F	135± 3.0	133±10.5	**122**±6.8**	128±4.1	132±4.6
Body Weights (g) (3 K)	Day 29	M	183±8.8	182±8.1	**168*±15.1**	**172***±9.5	184±6.3
Body Weights (g) (1)	Day 33	M	223±10.3	224 ±10.5	**208*±15.7**	212±11.3	227±8.5
Body Weights (g) (1)	Day 36	M	253±11.6	254±11.5	**237*±17.3**	241±12.6	259±10.4
Body Weights (g) (1)	Day 43	M	318±14.6	324±15.5	**300*±19.8**	304±17.0	325±12.1
Body Weights (g) (1)	Day 78	M	498±23.9	**533*±33.7**	481±28.0	494±40.3	524±24.6
Body Weights (g) (1)	Day 82	M	481±23.0	**523*±34.1**	469±30.3	482±42.9	511±24.1
Body Weights (g) (1)	Day 85	M	502±22.2	**547*±35.1**	485±30.5	504±42.2	537±27.2
Body Weights (g) (3 K)	Day 85	F	282±28.3	293±15.8	288±12.1	293±13.9	**302*±23.0**
Body Weights (g) (1)	Day 88	M	513±23.8	**559**±33.7**	498±31.2	514±40.1	545±25.3
Body weight change (g) (3 K)	Days 1 to 5	F	10±0.4	10±1.3	**8*±1.4**	10±0.7	9±0.8
Body weight change (g) (3 K)	Days 12 to 15	F	15±1.8	15±1.5	14±2.3	15±1.0	**17*±0.9**
Body weight change (g) (1)	Days 15 to 19	F	21±1.8	21±2.3	20± 1.6	**19* ±1.3**	**18** ±1.8**
Body weight change (g) (1)	Days 19 to 22	M	28±2.0	27±2.0	**25**±2.6**	**25**±1.6**	28 ±1.6
Body weight change (g) (1)	Days 19 to 22	F	22±1.0	22±3.3	**20*±2.2**	**19*±1.6**	21±1.6
Body weight change (g) (3 K)	Days 22 to 26	F	31±1.8	30±3.1	**28*±1.7**	29±2.2	30 ±2.6
Body weight change (g) (1)	Days 26 to 29	M	34±2.0	33±1.7	**31*±2.8**	**31*±2.5**	34±2.1
Body weight change (g) (1)	Days 40 to 43	F	6±2.9	9±4.1	8±4.1	7±6.3	**12* ±4.0**
Body weight change (g) (1)	Days 68 to 71	F	-1±5.9	7*±4.0	-1±5.5	-1±4.8	3±6.0

Table 6.3.1.4-1 Summary of the Statistically Significant Observations in 90-day Juvenile Rat Toxicity Study using Inbiose's 6'-SL Sodium Salt Ingredient

Parameters	Exposure	Sex	Dose Group (6'-SL sodium salt mg/kg bw/day)				
			0	1,500	2,500	4,000	Reference item, FOS (4,000)
Body weight change (g) (1)	Days 71 to 75	M	11±6.8	**19* ±5.3**	18 ±5.6	15 ±7.1	17 ±4.5
Body weight change (g) (1)	Days 1 to 15	M	44±2.2	43 ± 3.6	**39** ±3.3**	43±3.7	46±1.6
Body weight change (g) (3 K)	Days 15 to 29	M	119±7.6	120±7.0	**110*± 13.7**	**110*±7.1**	118±6.6
Body weight change (g) (1)	Days 29 to 57	F	88±17.3	95±10.2	98±9.2	97±10.4	**104* ±18.3**
Body weight change (g) (3 K)	Days 57 to 88	M	90±17.3	**121** ±9.3**	100±15.7	103±20.6	111 ±11.9
Body weight change (g) (1)	Days 1 to 88	M	493±23.7	**539**±33.0**	479 ±30.6	495 ±40.5	525 ±25.1
Final Body Weight/Food Consumption/Organ Weights (Mean values ± SD)							
Final body weight (g)	Days 89 to 90	M	485.0± 25.13	**532.1**±33.95**	476.9±32.59	491.7±41.19	518.7 ±23.61
Food Consumption (g/animal/day) (3)	Days 19/22	M	15.5±0.24	15.2±0.54	**13.8*± 0.88**	**14.0*± 0.45**	15.5±0.73
Food Consumption (g/animal/day) (3)	Days 47/50	M	33.0±0.52	30.7± 1.42	**29.6**± 1.29**	30.6±1.07	31.1±0.58
Food Consumption (g/animal/day) (3)	Days 61/64	M	33.1± 1.10	32.8±1.27	30.7±1.11	**30.0*±1.03**	32.7±1.41
Liver Mean weight (3)	Days 89 to 90	M	11.59±0.941	**12.94**±1.16**	12.02±0.674	11.62±0.749	11.69 ±0.798
Spleen Mean weight (3)	Days 89 to 90	M	1.20±0.071	**1.47*±0.253**	1.26±0.193	1.41±0.223	1.34 ±0.167
Testis Mean weight (3)	Days 89 to 90	M	3.45±0.236	3.72±0.218	3.63±0.323	3.58±0.152	**3.71**±1.02**
Thymus Mean weight (3)	Days 89 to 90	M	0.47650±0.038	**0.59260*±0.131**	0.48180±0.103	0.45178±0.081	0.52480±0.076
Ovaries Mean weight (1)	Days 89 to 90	F	0.12870±0.015	0.14860±0.013	0.14340±0.027	0.13833±0.024	**0.15130* ±0.016**
Motor Activity (Mean values ± SD)							
Rearing (3 K)	Week 11 to 12	F	214±41.0	201±32.4	187±48.6	**145*±62.6**	195±30.8
Thyroid Hormones (Mean values ± SD)							
Mean TSH (pg/mL) (1)	Week 12	F	426±179.0	569±251.3	**885**±339.6**	**873**±419.6**	**782*±273.1**

Table 6.3.1.4-1 Summary of the Statistically Significant Observations in 90-day Juvenile Rat Toxicity Study using Inbiose's 6'-SL Sodium Salt Ingredient

Parameters	Exposure	Sex	Dose Group (6'-SL sodium salt mg/kg bw/day)				
			0	1,500	2,500	4,000	Reference item, FOS (4,000)
Hematology (Mean values ± SD)							
RBC T/L (1)	PND 88	M	9.07±0.286	**8.65*±0.218**	9.06±0.442	**8.58*±0.229**	9.22±0.485
MCV (fL) (3)	PND 88	M	52.6±1.17	**54.0*±1.22**	53.4±1.27	54.0±1.62	53.3±0.98
MCHC (g/dL) (1)	PND 88	M	32.8±0.29	32.9±0.33	32.6±0.26	32.8±0.51	**32.4 *±0.51**
RTC (%)) (3)	PND 88	M	2.66±0.428	3.03±0.358	3.22±0.753	**3.32 *±0.494**	2.6±0.282
RTC (T/L) (1)	PND 88	M	0.24±0.037	0.26±0.030	**0.29*±0.065**	0.28±0.046	0.24±0.018
FIB (g/L) (1)	PND 89	F	2.87±0.224	2.69±0.132	2.82±0.182	2.80±0.149	**2.63 *±0.169**
APTT (s) (1)	PND 88	M	17.6±0.97	17.4±0.91	18.2±1.61	**15.8 *±1.66**	16.3±1.35
Blood Biochemistry (Mean values ± SD)							
Na+ (mmol/L) (1)	PND 88	M	143.0±0.74	143.8±0.92	143.8±0.66	143.2±0.79	**143.8*±0.45**
Na+ (mmol/L) (1)	PND 89	F	141.2±0.71	140.8±0.79	141.3±0.92	**140.0*±**0.73	140.8±0.96
K+ (mmol/L) (1)	PND 88	M	4.06±0.203	3.97±0.181	**3.81*±0.227**	3.94±0.251	3.85±0.186
Cl- (mmol/L) (1)	PND 88	M	106.0±0.55	106.8±0.66	**105.1*±0.92**	**105.1*±0.45**	106.1±1.05
CHOL (mmol/L) (1)	PND 89	F	1.53±0.181	1.83±0.315	1.74±0.440	1.86±0.258	**2.00** ±0.257**
GLUC (mmol/L) (3 K)	PND 88	M	8.03±0.515	7.61±0.649	**6.48**±0.619**	7.59±0.885	**6.58**±0.293**
GLUC (mmol/L) (1)	PND 89	F	7.67±0.821	**6.57**±0.760**	6.97±0.654	6.95±0.596	6.96±0.686
TOT.BIL (µmol/L) (1)	PND 88	M	0.96±0.298	0.92±0.310	**0.25**±0.365**	0.99±0.355	0.60±0.409
ALP (U/L) (1)	PND 88	M	301±32.7	**362*± 47.2**	312±53.8	336 ±51.7	340 ±33.3

6'-SL = 6'-siayllactose; ALP = alkaline phosphatase; APTT = activated partial thromboplastin time; CHOL = total cholesterol; Cl- = chloride; F = female; FIB = fibrinogen; GLUC = glucose; K^+ = potassium; M = male; MCHC = mean cell hemoglobin concentration; MCV = mean cell volume; Na^+ = sodium; PND = Post-natal Day; RBC = erythrocytes; RTC = reticulocytes; SD = standard deviation; TOT.BIL = total bilirubin; TSH = thyroid stimulating hormone.

* P<0.05, ** P<0.01

(1) DUNNETT TEST

(3) DUNN TEST

(B) BARTLETT TEST P<0.01

(K) KOLMOGOROV-LILLIEFORS TEST P<0.01

Assigned control group(s) : 1.

6.3.1.5 Toxicokinetics

Two toxicokinetic studies were conducted with Inbiose's 6'-SL sodium salt in juvenile and young adult rats.

In the preliminary juvenile rat study, 6 satellite pups/sex/group were evaluated for toxicokinetic assessment in plasma and urine. Animals received 6'-SL sodium salt at dose levels of 3,000, 4,000, or 5,000 mg/kg bw/day *via* gavage for 21 days (from PND 7 to PND 27). Blood samples for the determination of plasma levels of 6'-SL were collected on PND 27 at 0, 1, 2, 4, 6, and 24 hours. The traces of 6'-SL were noted, as plasma and urine concentrations of 6'-SL were above the lower limit of quantification (LLOQ: 100 ng/mL for plasma and 500 ng/mL for urine) in 6 of 18 control plasma samples of females and 7 of 18 plasma samples of males and in all urine samples. This was more likely due to an endogenous presence of the 6'-SL in the blood circulation as compared to study of Vazquez *et al.* (2017). No obvious difference in plasma exposure was noted between juvenile males and females as the male to female maximum plasma concentration (C_{max}) and AUC_{0-t} ratios were close to 1, with the exception of the animals treated at 4,000 mg/kg bw/day. When considering C_{max} and AUC_{0-t} ratios, males seemed to be more exposed to the test item than females. This could be also due to the high variability observed in this group. When considering the urinary results, the males had higher 6'-SL concentrations than females at all dose levels. Based on dose-normalized AUC_{0-t}, a dose-proportional increase in plasma 6'-SL exposure was noted in female SD rats over the range of administered dose, while a more than dose-proportional increase was observed in males from 3,000 to 4,000 mg/kg bw/day and a less than dose-proportional increase from 4,000 to 5,000 mg/kg bw/day. When considering the overnight urinary collection, a clear more than dose-proportional concentration increase occurred, thus suggesting either a dose-related change in the renal clearance of 6'-SL or an incomplete period of collection or the combination of both hypotheses.

In the 90-day sub-chronic juvenile rat study, 4 groups of rats (3/sex/group) received 6'-SL sodium salt daily *via* gavage, at the dose level of 0, 1,500, 2,500 or 4,000 mg/kg bw/day, from PND 7 to at least PND 90. Blood samples for the determination of plasma levels of the test item were collected at the end of the treatment period on PND 87 (control group 1) or 89 (6'-SL sodium salt-treated groups 2 to 4) at 1, 2, 4, 6, and 24 hours after administration. The control animal blood samples were taken on a different day to those of treated animals to avoid any possibility of contamination. Urine samples were collected from animals at the end of the treatment period. Similar to the preliminary study, the traces of 6'-SL sodium salt were observed in control plasma samples collected before administration of the control item (179 ng/mL in 1 male and at 4 hours after administration of the control item (115 ng/mL in female rat) (LLOQ: 100 ng/mL). The traces (less than 10% of the C_{max} of the low-dose group) of 6'-SL sodium salt were observed in control urine samples in 2 control group females (17.0 µg/mL) (LLOQ: 10 µg/mL). Low but measurable endogenous levels of 6'-SL were already reported by Vazquez *et al.* (2017) in rats at baseline. Systemic exposure to 6'-SL sodium salt was achieved in all 6'-SL sodium salt-treated animals. The plasma concentration peak was observed 1 hour after administration. No marked differences in systemic exposure to 6'-Sialyllactose was observed between male and female rats. The male-to-female ratios ranged between 0.941 and 1.39 for C_{max} and 0.726 to 1.09 for area under the concentration-time curve (AUC). In the range of administered doses, the C_{max} and AUC_{0-t} increased proportionally with doses. The urine excretion was similar between male and female rats except in the low dose group (1,500 mg/kg bw/day) where higher excreted amount of 6'-Sialyllactose was observed in males. The male-to-female ratios were 2.96, 1.12, and 1.48 after 1,500, 2,500, and 4,000 mg/kg bwday administration, respectively.

6.3.1.6 *Summary of Studies Conducted with Inbiose's 6'-SL Sodium Salt*

Table 6.3.1.6-1 Summary of Toxicological Studies to Support the Safety of Inbiose's 6'-SL Sodium Salt

Type of Study	Species or Cell Type	Length of Study	6'-SL Dose and Route of Administration	Result	Reference
Studies Conducted with Inbiose 6'-SL Sodium Salt					
Acute oral toxicity study (OECD TG 425) (OECD, 2008)	Three female Crl:WI Wistar rats	Single dose *via* gavage followed by a 14-day observation period	5,000 mg/kg bw 6'-SL sodium salt dissolved in distilled water – the highest recommended dose	LD_{50} of 6'-SL was found to be greater than 5,000 mg/kg bw.	Tarcai (2020) [unpublished], final report
Bacterial reverse mutation test (OECD TG 471) (OECD, 1997)	*Salmonella* Typhimurium strains TA98 (pKM101), TA100 (pKM101), TA1535, and TA1537 and *Escherichia coli* strain WP2 uvrA	Plate incorporation assay and pre-incubation method	Up to 5,000 µg/plate (±S9)	6'-SL is non-mutagenic under the conditions of this test.	Chevallier (2020a) [unpublished], final report
In vitro mammalian chromosome aberration test (OECD TG 473) (OECD 2016b),	Human peripheral blood lymphocytes	3 and 20 h (-S9 mix) and 3 h (+ S9 mix)	0 (water for injection, vehicle) 125, 250, 500, 1,000, and 2,000 6'-SL sodium salt µg/mL (2,000 µg/mL being the highest recommended limit dose level	6'-SL did not induce chromosome aberrations in cultured peripheral blood human lymphocytes, either in presence of absence of S9 mix.	Chevallier (2020c) [unpublished], final report
In vitro mammalian cell micronucleus test (OECD TG 487) (OECD, 2016a)	Human peripheral blood lymphocytes	3-h treatment followed by 24-h recovery (±S9 mix) and 24-h continuous treatment with no recovery (-S9 mix)	0 (water for injection, vehicle) 125, 250, 500, 1,000, and 2,000 6'-SL sodium salt µg/mL (2,000 µg/mL being the highest recommended limit dose level	6'-SL sodium salt, did not induce any chromosome damage, or damage to the cell division Apparatus.	Chevallier (2020b) [unpublished], final report
Repeat dose toxicity study in Juvenile rats	Group of 8 male and 8 female juvenile SD rats Satellite group of 8 male and female juvenile SD rats	21 days from PND 7	0 (water for injection, vehicle) 3,000, 4,000, or 5,000 mg/kg bw/day by gavage	4,000 mg/kg bw/day was selected as the appropriate highest dose for the main 90-day study.	Spézia (2020) [unpublished], final report
Subchronic toxicity study (OECD TG 408) (OECD, 2018)	Groups of 10 male and 10 female neonatal SD, RjHan:SD (CD®) rats	90 days from PND 7	0 (water for injection, vehicle) 1,500, 2,500 or 4,000 mg/kg bw/day by gavage	NOAEL is 4,000 mg/kg bw/day of 6'-SL sodium salt.	Haag (2021) [unpublished], pre-final report

6'-SL = 6'-sialyllactose sodium salt; bw = body weight; h = hours; LD_{50} = median lethal dose; NOAEL = no-observed-adverse-effect level; OECD = Organisation for Economic Co-operation and Development; PND = Post-natal Day; S9 = metabolic activation mix; SD = Sprague-Dawley; TG = Test Guideline.

6.3.2 Studies Conducted with Other 6'-SL Sodium Salt Preparations

Pivotal safety data and information has been discussed previously and is hereby incorporated by reference to Part 6.4 of GRN 881 and Section VI Part C of GRN 922 (U.S. FDA, 2020a, 2021). Analytical data of Inbiose's 6'-SL sodium salt product establish the ingredient as chemically identical to its 6'-SL counterpart in human breast milk (see Section 2.1.1). Based on analytical data presented demonstrating that 6'-SL produced by Inbiose is of similar purity to 6'-SL preparations that have previously been concluded to be GRAS, studies characterizing the toxicity and safety of 6'-SL in animal models are considered relevant to the safety assessment of Inbiose's ingredient. The toxicological studies in GRN 881 and 922 are briefly summarized below in Table 6.3.2-1.

To note, these studies include a 6'-SL sodium salt ingredient produced by GeneChem Inc. *via* enzymatic synthesis (enzymes derived from a beta-D-galactosidase deficient *E. coli* BW25113).

Table 6.3.2-1 Summary of Toxicological Studies to Support the Safety of Inbiose's 6'-SL Sodium Salt

Type of Study	Species or Cell Type	Length of Study	6'-SL Dose and Route of Administration	Result	Reference
Studies Conducted with Glycom's 6'-SL (GRN 881) (U.S. FDA, 2020a)					
Bacterial reverse mutation test	*Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* strain WP2 uvrA (pKM101)	Plate incorporation assay and pre-incubation assay	Up to 5,000 µg/plate (±S9)	6'-SL is non-mutagenic at concentrations up to 5,000 µg/plate.	Phipps *et al.* (2019)
In vitro mammalian cell micronucleus test	Human lymphocytes	+S9 = 3 h -S9 = 3 and 24 h	500, 1,000, or 2,000 µg/mL	6'-SL is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL.	
14-day oral toxicity study	Groups of 8 male and 8 female neonatal rats	14 days	0 (water for irrigation), 4,000, or 5,000 mg/kg bw/day of 6'-SL, by gavage	5,000 mg/kg bw/day of 6'-SL is the highest dose selected in the subchronic study.	
90-day oral toxicity study	Groups of 10 male and 10 female neonatal Crl:CD(SD) rats	90 days	0 (water for irrigation), 1,000, 3,000, or 5,000 mg/kg bw/day 6'-SL, by gavage	NOAEL is 5,000 mg/kg bw/day of 6'-SL.	

Table 6.3.2-1 Summary of Toxicological Studies to Support the Safety of Inbiose's 6'-SL Sodium Salt

Type of Study	Species or Cell Type	Length of Study	6'-SL Dose and Route of Administration	Result	Reference
Studies Conducted with Jennewein's 6'-SL as Part of an HMO Mixture[a] (GRN 922) (U.S. FDA, 2021)					
Bacterial reverse mutation assay	*Salmonella* Typhimurium strains TA98, TA100, TA102, TA1535, and TA1537	Plate incorporation test and pre-incubation test	5, 10.0, 31.6, 100, 316, or 600 mg of the HMO mixture per plate containing 0.2, 0.4, 1.3, 4, 12.6, and 24 mg 6'-SL per plate	The HMO mixture, and the 6'-SL contained therein, was not mutagenic under the conditions tested.	Parschat *et al.* (2020)
In vitro micronucleus assay	Human peripheral blood lymphocytes	4 or 24 h (±S9)	7.5, 15, 30, and 60 mg HMO mixture/mL medium (equivalent to 0.3, 0.6, 1.2, and 2.4 mg 6'-SL/mL medium)	The HMO mixture was not genotoxic under the tested conditions at concentrations up to 60 mg/mL (2.4 mg/mL 6'-SL).	
Seven-day pilot dietary toxicity study	Groups of 5 female CD/Crl:CD rats	7 days	A control diet or the same diet containing 10% of an HMO mixture (equivalent to 0.4% 6'-SL)	No HMO-related differences in behavior, appearance, and consistency of the feces, bw, bw gain, or feed consumption were observed.	
90-day feeding study	Groups of 10 male and female CD/Crl:CD rats	90 days	A control diet or the same diet containing 10% of an HMO mixture (equivalent to 0.4% 6'-SL)	NOAEL for this study was 5.67 g/kg/day for male rats and 6.97 g/kg/day for the female rats. This resulted in a mean intake of 6'-SL of 0.23 g/kg/day in males and 0.28 g/kg/day in females.	

Table 6.3.2-1 Summary of Toxicological Studies to Support the Safety of Inbiose's 6'-SL Sodium Salt

Type of Study	Species or Cell Type	Length of Study	6'-SL Dose and Route of Administration	Result	Reference
21 day-neonatal piglet study	Groups of 6 male and female LD-2 Domestic Yorkshire Crossbred Swine (farm pig)	21 days	A control diet; or Oligosaccharide blend (2.8 g 2'-FL/L, 0.6 g 3-FL/L, 1.2 g LNT/L, 0.2 g 3'-SL/L, and 0.2 g 6'-SL/L) in the diet; or Oligosaccharide blend (3.9 g 2'-FL/L, 0.8 g 3-FL/L, 1.6 g LNT/L, 0.3 g 3'-SL/L, and 0.3 g 6'-SL/L) in the diet	The Oligosaccharide blend was well tolerated and did not produce adverse effects on the growth and development of the pigs. No Oligosaccharide blend-related mortalities occurred. The clinical pathology values and macroscopic and microscopic findings at necropsy did not reveal a relationship to treatment with the Oligosaccharide Blend at the concentrations evaluated. No adverse findings in gross or histopathology were noted.	Hanlon (2020)
Studies Conducted with GeneChem's 6'-SL					
Single dose acute toxicity study	Groups of 5 male and 5 female SD rats	Single dose	0 (purified water), 5,000, 10,000, 15,000, or 20,000 mg/kg bw/day 6'-SL by oral gavage	LD_{50} of 6'-SL was found to be greater than 20,000 mg/kg bw/day.	Gurung *et al.* (2018)
90-day oral toxicity study	Groups of 11 male and 11 female SD rats	90 days	0 (purified water), 1,000, 2,500, or 5,000 mg/kg bw/day of 6'-SL, by oral gavage	NOAEL is 5,000 mg/kg bw/day of 6'-SL.	
Bacterial reverse mutation test	*Salmonella* Typhimurium strains TA97, TA98, TA100, TA102 and TA1535	Plate incorporation assay and pre-incubation assay	Up to 5,000 µg/plate (±S9)	6'-SL is non-mutagenic under the conditions of this test.	
In vitro chromosome aberration test	CHL cells	-	225, 450, or 900 µg/mL (±S9)	6'-SL is neither clastogenic nor aneugenic under the conditions of this test.	
In vivo mammalian erythrocyte micronucleus test	Groups of 5 male and 5 female Kunming mice	Two successive days, approximately 18 h apart	Purified water (vehicle control) or 500, 1,000, or 2,000 mg/kg bw/day of 6'-SL by oral gavage	6'-SL is neither clastogenic nor aneugenic *in vivo*, under the conditions of this test.	

Table 6.3.2-1 Summary of Toxicological Studies to Support the Safety of Inbiose's 6'-SL Sodium Salt

Type of Study	Species or Cell Type	Length of Study	6'-SL Dose and Route of Administration	Result	Reference

2'-FL = 2'-fucosyllactose; 3'-SL = 3'-sialyllactose sodium salt; 3-FL = 3-fucosyllactose; 6'-SL = 6'-sialyllactose sodium salt; bw = body weight; CHL = Chinese hamster lung cells; GeneChem = GeneChem Inc.; Glycom = Glycom A/S; GRN = GRAS Notice; h = hours; HMO = human milk oligosaccharide; Jennewein = Jennewein Biotechnologie GmbH; LD_{50} = median lethal dose; LNT = lacto-*N*-tetraose; NOAEL = no-observed-adverse-effect level; S9 = metabolic activation mix; SD = Sprague-Dawley.
[a] HMO mixture = 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein using fermentation.

One additional study conducted with GeneChem's 6'-SL sodium salt was identified in the search of the scientific literature published after the submission of GRN 922, as described in Section 6.1 (U.S. FDA, 2021). Groups of twelve neonatal piglets (6/sex/group) were administered 0 (control formula) 300, 600, or 1,200 mg 6'-SL sodium salt/L for 21 days (Monaco *et al.*, 2020). On Days 1 to 5 of treatment, the piglets were provided 300 mL formula/kg body weight/day, which was increased to 330 mL formula/kg body weight/day from Day 6 onwards. Throughout the study, clinical observations were made and body weights recorded. Blood samples were taken on Days 8 and 22 of the study and urine samples were collected at necropsy. Organ samples (*i.e.*, spleen, stomach, kidney, heart, lung, liver) were collected, weighed, and fixed for histopathological analysis and the Mesenteric lymph nodes, pancreas, and gallbladder were collected and fixed (not weighed) at necropsy. The length and weight of the intestines were also recorded and samples were fixed for microscopic histological analyses. No significant adverse effects were noted in any growth parameters in piglets treated with 6'-SL sodium salt. Minor findings were noted in the macroscopic evaluation of tissues; however these changes were not significantly different from the control animals. 6'-SL administration did not have any effect on measured blood chemistry parameters. Overall, no dose-dependent effects were observed and clinical parameters remained within established reference ranges. The authors therefore concluded that 6'-SL sodium salt administration "*supported normal growth and development of piglets at concentrations of up to 1,200 mg/L*" (Monaco *et al.*, 2020).

6.4 Human Studies

No human studies conducted with 6'-SL were identified in GRN 881 and 922 (U.S. FDA, 2020a, 2021). However, many studies have been published that investigated the effects of supplementing infant formula with HiMOs, including 3'-SL sodium salt, the constitutional isomer of 6'-SL sodium salt. The weight of the available evidence (published clinical data) evaluating the safety and tolerance of HiMOs in infants supports the conclusion that 3'-SL sodium salt preparations are GRAS for use in infant formula at use levels of up to 0.28 mg/L. The summaries of these studies are incorporated by reference to previous GRAS conclusions, *i.e.*, GRN 766, 880, and 921 (Glycom A/S, 2019b; Jennewein Biotechnologie GmbH, 2020b; U.S. FDA, 2018b, 2020g,h). These studies in infants regarded the addition of 3'-SL sodium salt to infant formula as well-tolerated and did not indicate the manifestation of adverse effects related to growth or development. No additional studies were identified in the literature as being published subsequent to the most recent 3'-SL sodium salt GRAS Notice.

Similarly, no new studies of 6'-SL sodium salt administration in adults have been identified in the scientific literature since the most recent 6'-SL sodium salt GRAS Notice. Summaries of previously identified studies in adults are hereby incorporated by reference to previous GRAS conclusions, *i.e.*, GRN 766, 880, and 921 (U.S. FDA, 2018b, 2020g,h), which support well-tolerated safe use levels of up to 20 g/day of 3'-SL sodium salt in adults.

6.5 Allergenicity

The potential allergenic activity of the recombinant proteins expressed in *E. coli K-12 MG1655* INB-6SL_01 and INB-6SL_02 was assessed by using the Allergen Online Tool (V20, released on 10 February 2020) of the University of Nebraska – Lincoln (FARRP, 2020). The database contained 2,171 putative allergen sequences. Potential allergenicity was evaluated by scanning each possible 80-amino acid segment of the recombinant protein (sliding window) to the database, and therefore looking for matches of at least 35% identity. No sequence alerts from potential allergens were identified for the recombinant proteins in INB-6SL_01 and INB-6SL_02.

Since lactose is used as substrate in the 6'-SL production process and small amounts of residual lactose are present in the final product, the label “contains milk”, in accordance with the requirements of the Food Allergy, Labelling and Consumer Protection Act of 2004, must be added.

6.6 General Recognition

As discussed, the use of 6'-SL sodium salt as an ingredient in non-exempt term infant formula at levels up to 0.4 g/L and in various conventional food products has been evaluated by multiple experts, qualified through scientific training and experience, in the safety evaluation of food and infant formula ingredients (GRN 881 and 922) (U.S. FDA, 2020a, 2021). The use of 6'-SL sodium salt in infant formula at concentrations up to 0.4 g/L and various food products also has been the subject of comprehensive evaluations by multiple authoritative bodies, including EFSA (EFSA, 2020). As Inbiose has demonstrated that 6'-SL sodium salt manufactured by the company is qualitatively and quantitatively highly similar to 6'-SL sodium salt ingredients that have been the subject of previous GRAS evaluations and global novel food approvals and is intended for use in the same foods and at the same use levels as those concluded to be GRAS, conclusions on the safety of 6'-SL sodium salt for these uses issued by various experts and scientific bodies forms a basis for general recognition of Inbiose’s GRAS conclusion. Convening of a GRAS Panel was therefore not considered necessary to support a GRAS conclusion on the basis that this HMO ingredient has been evaluated by multiple GRAS Panels and authoritative bodies, including the U.S. FDA and EFSA.

6.7 Conclusion

Based on the above data and information presented herein, Inbiose has concluded that 6'-SL sodium salt is GRAS, on the basis of scientific procedures, for use in non-exempt term infant formula and specified conventional food and beverage products as described in Section 1.3.

6'-SL sodium salt 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

Austin S, De Castro CA, Benet T, Hou Y, Sun H, Thakkar SK, et al. (2016). Temporal change of the content of 10 oligosaccharides in the milk of Chinese urban mothers. Nutrients 8(6):346 [22pp]. DOI:10.3390/nu8060346. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Austin S, De Castro CA, Sprenger N, Binia A, Affolter M, Garcia-Rodenas CL, et al. (2019). Human milk oligosaccharides in the milk of mothers delivering term versus preterm infants. Nutrients 11(6):1282 [17pp, plus supplementary data]. DOI:10.3390/nu11061282. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Azad MB, Robertson B, Atakora F, Becker AB, Subbarao P, Moraes TJ, et al. (2018). Human milk oligosaccharide concentrations are associated with multiple fixed and modifiable maternal characteristics, environmental factors, and feeding practices. J Nutr 148(11):1733-1742 [plus supplementary data]. DOI:10.1093/jn/nxy175. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, et al. (1997). The complete genome sequence of *Escherichia coli* K-12. Science 277(5331):1453-1462 [plus supplementary data]. DOI:10.1126/science.277.5331.1453.

Brand-Miller JC, McVeagh P, McNeil Y, Messer M (1998). Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. J Pediatr 133(1):95-98. DOI:10.1016/S0022-3476(98)70185-4.

CDC (2015). *National Health and Nutrition Examination Survey (NHANES): 2013-2014*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/search/nhanes13_14.aspx [Page last updated: October 30, 2015].

CDC (2016). *National Health and Nutrition Examination Survey (NHANES): 2013-2014 – Dietary Data*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: https://wwwn.cdc.gov/nchs/nhanes/Search/DataPage.aspx?Component=Dietary&CycleBeginYear=2013 [Date published: September 2016].

Chevallier K (2020a) [unpublished]. *Bacterial Reverse Mutation Test, Test Item 6'-Sialyllactose (6´-SL) Sodium Salt: Final Report*. (Study No.47614 MMJ). Charles River Laboratories Evreux (formerly Citoxlab-FR Evreux).

Chevallier K (2020b) [unpublished]. *Test Item 6'-Sialyllactose (6´-SL) Sodium Salt - In Vitro Micronucleus Test in Cultured Human Lymphocytes: Final Report*. (Study No. 47615 MNH). Charles River Laboratories Evreux (formerly Citoxlab-FR Evreux.

Chevallier K (2020c) [unpublished]. *Test Item 6'-Sialyllactose (6´-SL) Sodium Salt - In Vitro Mammalian Chromosome Aberration Test in Cultured Human Lymphocytes: Final Report*. (Study No. 47616 MLH). Charles River Laboratories Evreux (formerly Citoxlab-FR Evreux.

Coulet M, Phothirath P, Constable A, Marsden E, Schilter B (2013). Pre-clinical safety assessment of the synthetic human milk, nature-identical, oligosaccharide Lacto-*N*-neotetraose (LNnT). Food Chem Toxicol 62:528-537. DOI:10.1016/j.fct.2013.09.018.

Datsenko KA, Wanner BL (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci USA 97(12):6640-6645. DOI:10.1073/pnas.120163297.

EFSA (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA Panel on Additives and Products or Substances Used in Animal Feed/FEEDAP) (Question no EFSA-Q-2016-00069 and EFSA-Q-2017-00211, adopted: 21 February 2018 by European Food Safety Authority). EFSA J 16(3):5206 [24pp]. DOI:10.2903/j.efsa.2018.5206. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/5206.

EFSA (2020). Safety of 6'-Sialyllactose (6'-SL) sodium salt as a novel food pursuant to Regulation (EU) 2015/2283. (EFSA Panel on Nutrition, Novel Foods and Food Allergens/NDA) (Question no: EFSA-Q-2019-00169; adopted: 23 March 2020 by European Food Safety Authority). EFSA J 18(5):6097 [23pp]. DOI:10.2903/j.efsa.2020.6097. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/6097.

Engfer MB, Stahl B, Finke B, Sawatzki G, Daniel H (2000). Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr 71(6):1589-1596. DOI:10.1093/ajcn/71.6.1589.

FARRP (2020). *AllergenOnline Version 20: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org [Released: February 10, 2020].

Frazier KS, Seely JC, Hard GC, Betton G, Burnett R, Nakatsuji S, et al. (2012). Proliferative and nonproliferative lesions of the rat and mouse urinary system. Toxicol Pathol 40(4, Suppl.):14S-86S. DOI:10.1177/0192623312438736.

Freddolino PL, Amini S, Tavazoie S (2012). Newly identified genetic variations in common *Escherichia coli* MG1655 stock cultures. J Bacteriol 194(2):303-6. DOI:10.1128/JB.06087-11.

FSANZ (2021). *A1155 – 2'-FL and LNnT in Infant Formula and Other Products*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/code/applications/Pages/A1155.aspx [Last updated: 1 February 2021].

Glycom A/S (2019a). *GRAS Notice for 6'-Sialyllactose Sodium Salt (6'-SL)*. (GRN No. 881). Prepared by Hørsholm, Denmark: Glycom A/S. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=881 [Feb. 24, 2020 - FDA response - no questions].

Glycom A/S (2019b). *GRAS Notice for 3'-Sialyllactose Sodium Salt (3'-SL)*. (GRN No. 880). Prepared by Hørsholm, Denmark: Glycom A/S. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=880 [Feb. 21, 2020 - FDA response - no questions].

Gnoth MJ, Kunz C, Kinne-Saffran E, Rudloff S (2000). Human milk oligosaccharides are minimally digested in vitro. J Nutr 130(12):3014-3020. DOI:10.1093/jn/130.12.3014.

Gurung RB, Kim DH, Kim L, Lee AW, Wang Z, Gao Y (2018). Toxicological evaluation of 6'-sialyllactose (6'-SL) sodium salt. Regul Toxicol Pharmacol 95:182-189. DOI:10.1016/j.yrtph.2018.03.010.

Haag V (2021) [unpublished]. *6'-Sialyllactose (6'-SL) Sodium Salt - Toxicity Study by Oral Route (Gavage) in Juvenile Rats: Pre-Final Report*. (Study No. 47619 RSR). Charles River Laboratories Evreux (formerly Citoxlab-FR Evreux.

Hanlon PR (2020). A safety evaluation of mixed human milk oligosaccharides in neonatal farm piglets. Toxicol Res Appl 4 [8pp]. DOI:10.1177/2397847320971255.

Jennewein Biotechnologie GmbH (2020a). *GRAS Determination for the Use of 6'-Sialyllactose Sodium Salt in Non-Exempt Term Infant Formula*. (GRN No. 922). Prepared by Rockville (MD): Spherix Consulting Group, Inc. Prepared for Reinbreitbach, Germany: Jennewein Biotechnologie, GmgH. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=922 [Apr. 23, 2021 - FDA response - no questions].

Jennewein Biotechnologie GmbH (2020b). GRAS Determination for the Use of 3'-Sialyllactose Sodium Salt in Non-Exempt Term Infant Formula. (GRN No. 921). Prepared by Rockville (MD): Spherix Consulting Group. Prepared for Rheinbreitbach, Germany: Jennewein Biotechnologie GmgH. Submitted to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=921 [Oct. 30, 2020 - FDA response - no questions].

Jokinen MP (1990). Urinary bladder, ureter, and urethra. In: Boorman GA, Eustis SL, Elwell MR, Montgomery CA, MacKenzie WF, editors. *Pathology of the Fischer Rat: Reference and Atlas*. San Diego (CA): Academic Press Inc., pp. 109-126.

Kjærulff L (2014). *NMR Structural Studies of Oligosaccharides and Other Natural Products* [PhD Thesis]. Lyngby, Denmark: Technical University of Denmark. Available at: https://orbit.dtu.dk/en/publications/nmr-structural-studies-of-oligosaccharides-and-other-natural-prod.

Kunz C, Meyer C, Collado MC, Geiger L, García-Mantrana I, Bertua-Ríos B, et al. (2017). Influence of gestational age, secretor and Lewis blood group status on the oligosaccharide content of human milk. J Pediatr Gastroenterol Nutr 64(17):789-798. DOI:10.1097/MPG.0000000000001402. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Larsson MW, Lind MV, Laursen RP, Yonemitsu C, Larnkjær A, Mølgaard C, et al. (2019). Human milk oligosaccharide composition is associated with excessive weight gain during exclusive breastfeeding-an explorative study. Front Pediatr 7:Article 297 [14pp, plus supplementary tables]. DOI:10.3389/fped.2019.00297. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, et al. (2017). What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. Am J Clin Nutr 105(5):1086-1100 [plus supplementary data]. DOI:10.3945/ajcn.116.139980. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

McJarrow P, Radwan H, Ma L, MacGibbon AKH, Hashim M, Hasan H, et al. (2019). Human milk oligosaccharide, phospholipid, and ganglioside concentrations in breast milk from United Arab Emirates mothers: results from the MISC cohort. Nutrients 11(10):2400 [14pp]. DOI:10.3390/nu11102400. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Monaco MH, Kim DH, Gurung RB, Donovan SM (2020). Evaluation of 6'-sialyllactose sodium salt supplementation to formula on growth and clinical parameters in neonatal piglets. Nutrients 12(4):1030 [12pp]. DOI:10.3390/nu12041030.

Nijman RM, Liu Y, Bunyatratchata A, Smilowitz JT, Stahl B, Barile D (2018). Characterization and quantification of oligosaccharides in human milk and infant formula. J Agric Food Chem 66(26):6851-6859. DOI:10.1021/acs.jafc.8b0151. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

OECD (1992). *Safety Considerations for Biotechnology 1992*. Paris, France: Organisation for Economic Co-Operation and Development (OECD). Available at: http://www.oecd.org/dataoecd/8/3/2375496.pdf.

OECD (1997). Bacterial reverse mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 471) [updated & adopted: 21 July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://www.oecd-ilibrary.org/fr/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en [Latest version updated & adopted: 26 June 2020].

OECD (2008). Acute oral toxicity: up-and-down procedure (UDP). In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 425) [Updated & Adopted: 3 October 2008]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-425-acute-oral-toxicity-up-and-down-procedure_9789264071049-en.

OECD (2016a). *In Vitro* mammalian cell micronucleus test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 487) [Updated & Adopted: 29 July 2016]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-487-in-vitro-mammalian-cell-micronucleus-test_9789264264861-en;jsessionid=58ip5ibjcf4kc.x-oecd-live-02.

OECD (2016b). *In Vitro* mammalian chromosome aberration test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 473) [Updated & Adopted: 29 July 2016]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://www.oecd-ilibrary.org/environment/test-no-473-in-vitro-mammalian-chromosomal-aberration-test_9789264264649-en.

OECD (2018). Repeated dose 90-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 408) [Updated & Adopted: 27 June 2018]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en.

OECD (2020). Bacterial reverse mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 471) [Updated & Adopted: 26 June 2020]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://www.oecd-ilibrary.org/docserver/9789264071247-en.pdf?expires=1609779422.

Paganini D, Uyoga MA, Kortman GAM, Boekhorst J, Schneeberger S, Karanja S, et al. (2019). Maternal human milk oligosaccharide profile modulates the impact of an intervention with iron and galacto-oligosaccharides in Kenyan infants. Nutrients 11(11):2596 [17pp, plus supplementary data]. DOI:10.3390/nu11112596. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Parschat K, Oehme A, Leuschner J, Jennewein S, Parkot J (2020). A safety evaluation of mixed human milk oligosaccharides in rats. Food Chem Toxicol 136:Article 111118 [12pp, plus supplementary tables]. DOI:10.1016/j.fct.2020.111118.

Peleg M, Normand MD, Corradini MG (2012). The Arrhenius equation revisited. Crit Rev Food Sci Nutr 52(9):830-851. DOI:10.1080/10408398.2012.667460.

Phipps KR, Baldwin NJ, Lynch B, Stannard DR, Šoltésová A, Gilby B, et al. (2019). Toxicological safety evaluation of the human-identical milk oligosaccharide 6'-sialyllactose sodium salt [plus supplementary data]. J Appl Toxicol 39(10):1444-1461 [plus supplementary tables]. DOI:10.1002/jat.3830.

Rudloff S, Kunz C (2012). Milk oligosaccharides and metabolism in infants. Adv Nutr 3(3, Suppl.):398S-405S. DOI:10.3945/an.111.001594.

Samuel TM, Binia A, de Castro CA, Thakkar SK, Billeaud C, Agosti M, et al. (2019). Impact of maternal characteristics on human milk oligosaccharide composition over the first 4 months of lactation in a cohort of healthy. Sci Rep 9(1):11767 [10pp, plus supplementary data]. DOI:10.1038/s41598-019-48337-4. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Snoeck N, De Mol ML, Van Herpe D, Goormans A, Maryns I, Coussement P, et al. (2019). Serine integrase recombinational engineering (SIRE): A versatile toolbox for genome editing. Biotechnol Bioeng 116(2):364-374. DOI:10.1002/bit.26854.

Spézia F (2020) [unpublished]. *Test Item 6'-Sialyllactose (6'-SL) Sodium Salt, Preliminary Toxicity Study by Oral Route (Gavage) In Juvenile Rats: Final Report.* (Study No. 47618 RSR). Charles River Laboratories Evreux (formerly Citoxlab-FR Evreux.

Sprenger N, Lee LY, De Castro CA, Steenhout P, Thakkar SK (2017). Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study. PLoS ONE 12(2):e0171814 [15pp, plus supplementary data]. DOI:10.1371/journal.pone.0171814. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Tarcai Z (2020) [unpublished]. *6´-Sialyllactose (6´-SL) Sodium Salt: Acute Oral Toxicity Study in Rats (Up and Down Procedure): Final Report.* (Study No. 19/300-001P). Charles River Laboratories Hungary, Veszprém (formerly Citoxlab- Hungary, Veszprém).

Thurl S, Munzert M, Boehm G, Matthews C, Stahl B (2017). Systematic review of the concentrations of oligosaccharides in human milk. Nutr Rev 75(11):920-933. DOI:10.1093/nutrit/nux044. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

Tonon K, de Morais MB, Abrão ACFV, Miranda A, Morais TB (2019). Maternal and infant factors associated with human milk oligosaccharides concentrations according to secretor and Lewis phenotypes. Nutrients 11(6):1358 [23pp, plus supplementary tables]. DOI:10.3390/nu11061358. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

U.S. EPA (1997). *Escherichia Coli K-12 Final Risk Assessment: Attachment I--Final Risk Assessment of Escherichia Coli K-12 Derivatives*. Washington (DC): U.S. Environmental Protection Agency (U.S. EPA), Biotechnology Program under the Toxic Substances Control Act (TSCA). Available at: https://www.epa.gov/sites/production/files/2015-09/documents/fra004.pdf [Last updated on September 27, 2012].

U.S. FDA (2018a). *Agency Response Letter GRAS Notice No. GRN 749 [2'-O-fucosyllactose, Wilmington (DE): DuPont Nutrition & Health]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=749 [Apr. 23, 2018 - FDA response - no questions].

U.S. FDA (2018b). *Agency Response Letter GRAS Notice No. GRN 766 [3'-sialyllactose sodium salt, Daejeon, Republic of Korea: GeneChem, Inc.]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=766 [Sep. 26, 2018; Correction: May 7, 2019 - FDA response - no questions].

U.S. FDA (2020a). *Agency Response Letter GRAS Notice No. GRN 881 [6'-sialyllactose sodium salt: Hørsholm, Denmark: Glycom A/S]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=881 [Feb. 24, 2020; Correction: Apr. 13, 2020 - FDA response - no questions].

U.S. FDA (2020b). Part 170—Food additives. Section §170.3—Definitions. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs* (Food and Drug Administration). Washington (DC): U.S. Food and Drug Administration (U.S. FDA), U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/ [current to 4-1-20, last updated 4-1-19].

U.S. FDA (2020c). Part 101—Food labeling. §101.12—Reference amounts customarily consumed per eating occasion. In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs*. (U.S. Food and Drug Administration). Washington (DC): U.S. Food and Drug Administration (U.S. FDA), U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/.

U.S. FDA (2020d). Part 170—Food additives. Section §170.30—Eligibility for classification as generally recognized as safe (GRAS). In: *U.S. Code of Federal Regulations (CFR). Title 21: Food and Drugs (U.S. Food and Drug Administration)*. Washington (DC): U.S. Food and Drug Administration (U.S. FDA), U.S. Government Printing Office (GPO). Available at: https://www.govinfo.gov/app/collection/cfr/ [current to 4-1-20, last updated 4-1-19].

U.S. FDA (2020e). *Agency Response Letter GRAS Notice No. GRN 897 [2'-O-fucosyllactose, Wilmington (DE): DuPont Nutrition and Health]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=897 [Jun. 12, 2020 - FDA response – no questions].

U.S. FDA (2020f). *Agency Response Letter GRAS Notice No. GRN 951 [3-fucosyllactose, Wilmington (DE): DuPont Nutrition and Biosciences]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=951 [Pending as of: Dec. 17, 2020].

U.S. FDA (2020g). *Agency Response Letter GRAS Notice No. GRN 880 [3'-sialyllactose sodium salt: Hørsholm, Denmark: Glycom A/S]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=880 [Feb. 21, 2020; Correction: Apr. 13, 2020 - FDA response - no questions].

U.S. FDA (2020h). *Agency Response Letter GRAS Notice No. GRN 921 [3'-sialyllactose sodium salt, Reinbreitbach, Germany: Jennewein Biotechnologie GmgH]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=921 [Oct. 30, 2020 - FDA response - no questions].

U.S. FDA (2021). *Agency Response Letter GRAS Notice No. GRN 922 [6'-sialyllactose sodium salt, Reinbreitbach, Germany: Jennewein Biotechnologie GmgH]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=922 [Apr. 23, 2021 - FDA response - no questions].

USDA (2016). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2013-2014.* Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release [Last Modified: October 13, 2016].

Vazquez E, Santos-Fandila A, Buck R, Rueda R, Ramirez M (2017). Major human milk oligosaccharides are absorbed into the systemic circulation after oral administration in rats. Br J Nutr 117(2):237-247. DOI:10.1017/S0007114516004554.

Wejryd E, Martí M, Marchini G, Werme A, Jonsson B, Landberg E, et al. (2018). Low diversity of human milk oligosaccharides is associated with necrotising enterocolitis in extremely low birth weight infants. Nutrients 10(10):1556. DOI:10.3390/nu10101556. Cited In: Jennewein Biotechnologie GmbH, 2020a [GRN 922].

From: Joeri Beauprez
To: Morissette, Rachel
Cc: Kamila Solak - Inbiose
Subject: [EXTERNAL] Re: questions for GRN 001075
Date: Monday, January 23, 2023 7:48:21 AM
Attachments: image014.png
image015.png
image016.png
image017.png
image018.png
image019.png
Outlook-rbqvykc0.png
Response letter_FDA_GRN 001075_6SL_Inbiose feedback.pdf

Dear Dr Morissette,

Thank you for the letter regarding GRN001075. Please find in attachment our response letter with the requested answers.

Feel free to contact us if you would have any further questions.

Kind regards,
Joeri

Joeri Beauprez, PhD

inbiose
specialty carbohydrates

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RPR Gent: 0534 887 593

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: 11 January 2023 19:44
To: Joeri Beauprez <Joeri.Beauprez@inbiose.com>
Subject: questions for GRN 001075

Dear Dr. Beauprez,

Please see below our questions for GRN 001075. We request that you provide responses within 10 business days. If you need more time, please let me know.

Best regards,

Rachel

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

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

FDA U.S. FOOD & DRUG ADMINISTRATION

I GOT MY COVID-19 VACCINATION

Questions for GRN 001075:

1. Please specify the protein base(s) of the non-exempt infant formula(s) to which you intend to add 6′-sialyllactose sodium salt (6′-SL).

2. In Table 2.3.1-1 on page 14 of the notice, a specification for "6′-SL sodium salt" is provided. Please clarify whether this specified limit includes the sodium component of the ingredient or if the limit is for 6′-SL only.

3. A specification for "sum of other carbohydrates" is provided in Table 2.3.1-1. Footnote b to Table 2.3.1-1 states that the other carbohydrates include sialic acid, lactose, and other carbohydrates. Footnote b to Table 2.3.2-1 on page 17 of the notice states the other carbohydrates may also include sialyllactulose. Please identify all other carbohydrates that may be present in 6′-SL sodium salt and that are included in this specification.

4. On page 35 of the notice, Inbiose states:

> "No effects on mean body weight, mean body weight change, mean food consumption, and on mean hematology or blood biochemistry parameters, up to 5,000 mg/kg bw/day were observed."

Inbiose goes on to state:

"As the cause of death of 2 animals could not be clearly established, 4,000 mg/kg bw/d was considered to be the acceptable high-dose level for the 90-day subchronic study."

The implication is that the 2 animal deaths with unknown causes that Inbiose refers to occurred at the 5,000 mg/kg bw/d group, but this was not specifically stated. Please clarify and provide a short rationale as to why 4,000 mg/kg bw/d rather than 5,000 mg/kg bw/d was chosen for the highest dose in your 90-day repeat dose oral toxicity study.

5. On pages 37 and 38, Inbiose states:

> "The thyroid hormone levels were considered to be unaffected by treatment with the test or reference item. The statistically significant higher mean thyroid stimulating hormone (TSH) levels in females given 6'-SL sodium salt at 2,500 or 4,000 mg/kg bw/d or FOS were not considered to be treatment-related as all individual values remained within the range of historical control data, and as these differences, which were not sex- or dose-related, did not correlate with any microscopic changes in the pituitary nor in the thyroid gland."

We note that previous 90-day repeated dose oral toxicity studies with 6'-SL (i.e., Phipps et al. 2019 and Gurung et al. 2018) did not measure thyroid hormone levels due to lack of statistical differences noted in thyroid gland and pituitary gland weights. Please confirm that in your unpublished study with your article of commerce, there was no statistically significant differences in thyroid and pituitary glands or in thyroid hormones (T3 and T4), which further corroborates your point that observed differences in TSH levels are not a safety concern.

6. Inbiose discusses in Section 6.3.1.5 on page 42 of the notice the results of their unpublished studies with respect to toxicokinetics of 6'-SL oral exposure in rats. Please provide a short narrative describing why the results you reported would not be considered counter to your GRAS conclusion.

7. On page 24 of the notice, Inbiose states that an updated comprehensive search of the publicly available scientific literature was conducted through March 2021. Please provide an updated literature search that explains how any newly found publicly available information still supports your GRAS conclusion. We note that, although Inbiose states on page 47 that no 6'-SL study in adult humans has been identified, we found a study that may be relevant to your safety assessment (Kim et al., 2022 Reg. Tox. Pharm. 129: 105110).

January 23, 2023

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Regarding: Response to FDA Questions related to GRAS Notice No. GRN 001075

Dear Dr. Morissette,

In reference to your email dated January 11, 2023, regarding Inbiose's GRAS Notice GRN 001075 for the intended uses of 6´-sialyllactose sodium salt (6'-SL), I am pleased to provide you with our responses to the Agency's questions in the following document.

I trust that all of your questions and comments are adequately addressed, below, and meet the Agency's expectations. If further clarification or any additional information is required as part of this GRAS Notice, please do not hesitate to let me know.

Kind regards,

Joeri Beauprez
Chief Scientific Officer

Question 1. Please specify the protein base(s) of the non-exempt infant formula(s) to which you

Inbiose is the bulk ingredient manufacturer of this ingredient, and therefore does not control the specific protein source that infant formula manufacturers may choose to use during the formulation of end use products. Protein sources used in the manufacture of infant formulae are defined by the infant formula manufacturer. Therefore, it is reasonable to expect that Inbiose's HMO ingredients, including 6'-SL, may be used in any of the available protein bases (*e.g.*, milk, soy, whey) that are currently used to manufacture non-exempt infant formula products.

Question 2. In Table 2.3.1-1 on page 14 of the notice, a specification for "6'-SL sodium salt" is

Inbiose confirms that the specified parameter limit for ''6'-SL sodium salt'' described in Table 2.3.1-1 on page 14 of the notice refers to the 6'-SL and sodium components. The values indicated in Table 2.3.2-1 for the parameter ''6'-SL sodium salt'' (page 16 of the notice) also include the sodium component.

Question 3. A specification for "sum of other carbohydrates" is provided in Table 2.3.1-1. Footnote b to Table 2.3.1-1 states that the other carbohydrates include sialic acid, lactose, and other carbohydrates. Footnote b to Table 2.3.2-1 on page 17 of the notice states the other carbohydrates may also include sialyllactulose. Please identify all other carbohydrates that may be present in 6'-SL sodium salt and that are included in this specification.

Thank you for identifying this inconsistency between the footnotes attached to the "sum of other carbohydrates" parameter included in Tables 2.3.1-1 and 2.3.2-1 of the notice. Both footnotes are meant to indicate that the ''sum of other carbohydrates'' parameter would predominantly include sialic acid and lactose, as well as other carbohydrates such as sialyllactulose. However, Inbiose would like to clarify that this specification parameter for "sum of other carbohydrates" refers to all carbohydrates present in the final ingredient except for 6'-SL. This parameter is measured using an HPAEC-PAD method, which is used to express the ''other carbohydrates'' as area %. As such, while ''other carbohydrates'' in Inbiose's 6'-SL includes predominantly sialic acid and lactose (each also listed separately in the specification as ≤5 % DM), this parameter may also include traces of other carbohydrates, such as *N*-acetylglucosamine (glcNAc), sucrose, sialyllactulose, glucose, and galactose. These carbohydrates are commonly present in a range of other HMOs (including other 6'-SL preparations) that have been notified to the U.S. FDA without objection from the agency ,*e.g.*, GRN 833 (list of 'other carbohydrates', page 78), GRN 881 (Table 2.3.1-1 Specifications for 6'-SL, page 14), GRN 922 (Table 3 Product Specifications and Batch Data for 6'-Sialyllactose, page 19; Table 8. Comparison of Jennewein's 6'-Sialyllactose Sodium Salt With the 6'-Sialyllactose Sodium Salt Tested Phipps et al. (2019) and That Supports GRN 881, page 35), GRN 923 (Table 3. Product Specifications and Batch Data for Lacto-N-tetraose, page 23; list of '"other carbohydrates', page 112), and GRN 1016 (Table 2. Product Specifications and Batch Data for 6'-Sialyllactose, page 15; list of '"other carbohydrates', page 83, therefore not expected to pose any safety concerns.

Inbiose would also like to highlight that the improvements performed in the second-generation strain resulted in a purer 6'-SL product, that contains a lower number of other carbohydrates specified above.

Question 4. On page 35 of the notice, Inbiose states:

> "No effects on mean body weight, mean body weight change, mean food consumption, and on mean hematology or blood biochemistry parameters, up to 5,000 mg/kg bw/day were observed."

Inbiose goes on to state:

> "As the cause of death of 2 animals could not be clearly established, 4,000 mg/kg bw/d was considered to be the acceptable high-dose level for the 90-day subchronic study."

The implication is that the 2 animal deaths with unknown causes that Inbiose refers to occurred at the 5,000 mg/kg bw/d group, but this was not specifically stated. Please clarify and provide a short rationale as to why 4,000 mg/kg bw/d rather than 5,000 mg/kg bw/d was chosen for the highest dose in your 90-day repeat dose oral toxicity study.

Thank you for providing us with the opportunity to bring clarity regarding the animal deaths in the dose range finding study and the subsequent selection of the highest dose in the 90-day sub-chronic study. The dose levels for the sub-chronic 90-day study were selected based on the results from the preliminary oral (gavage) toxicity study conducted in juvenile rats. In this study, juvenile male and female Sprague-Dawley rats received Inbiose's 6'-SL sodium salt once daily, from PND 7 to PND 27 (21-day treatment period), at the dose levels of 3,000, 4,000 or 5,000 mg/kg body weight/day.

Throughout the study, there were 4 unscheduled deaths in test item-treated groups. Two of these deaths were recorded on PNDs 17 and 18, in a satellite female and a satellite male rat treated with 3,000 and 4,000 mg 6'-SL sodium salt/kg body weight/day, respectively. No necropsy examination was performed for the satellite animals in this dose range finding study. The other two other unscheduled deaths were recorded in principal animals treated with 5,000 mg 6'-SL sodium salt/kg body weight/day. The observations made in the pathology report at necropsy for each of the principal animals are presented below:

> *One male (principal) was found dead on PND 18 (approx. 2h after dosing). This pup had feces with a yellowish color on PND 16 and 17. At necropsy, the cecum was distended with gas, the colon/jejunum/ileum had an orange content; and the lungs/bronchi had diffuse red discoloration (considered to be agonal findings). At microscopic examination, there were increased slight mixed inflammatory cell infiltrates in the mucosa of cecum and colon.*
>
> *One female (principal) was found dead on PND 20 (in the morning, before treatment): this pup had feces with yellowish color on PND 15 and 16. At necropsy, the cecum had a red discoloration and the jejunum/ileum/stomach was distended with gas. There were no indications of any technical issues during the dosing procedure. At microscopic*

examination, all the gastro-intestinal tissues were autolytic and the relationship of these changes to test item administration was considered to be equivocal.

Overall, all 4 unscheduled deaths in the preliminary rat study occurred between PND 17-20 (*i.e.*, during the weaning period and changing of the diet). While the cause of the unscheduled deaths of the 2 animals could not be clearly established (and intestinal gross changes may have been involved), the mid-dose of 4,000 mg/kg body weight/day was still considered by the study director to be an acceptable high-dose level for the 90-day oral gavage study.

Question 5. On pages 37 and 38, Inbiose states:

> "The thyroid hormone levels were considered to be unaffected by treatment with the test or reference item. The statistically significant higher mean thyroid stimulating hormone (TSH) levels in females given 6'-SL sodium salt at 2,500 or 4,000 mg/kg bw/d or FOS were not considered to be treatment-related as all individual values remained within the range of historical control data, and as these differences, which were not sex- or dose-related, did not correlate with any microscopic changes in the pituitary nor in the thyroid gland."

We note that previous 90-day repeated dose oral toxicity studies with 6'-SL (i.e., Phipps et al. 2019 and Gurung et al. 2018) did not measure thyroid hormone levels due to lack of statistical differences noted in thyroid gland and pituitary gland weights. Please confirm that in your unpublished study with your article of commerce, there was no statistically significant differences in thyroid and pituitary glands or in thyroid hormones (T3 and T4), which further corroborates your point that observed differences in TSH levels are not a safety concern.

The 90-day sub-chronic study of Inbiose's 6'-SL sodium salt was conducted in accordance with OECD Test Guideline 408, which was updated in 2018 to add endocrine-sensitive endpoints and includes thyroid hormone analysis. Inbiose confirms that no statistical differences were noted in thyroid gland and pituitary gland weights at necropsy at any tested 6'-SL sodium salt dose levels. The thyroid hormone levels (T3 and T4) were not statistically significantly elevated and thus considered to be unaffected by treatment with 6'-SL sodium salt. A statistically significant increase in mean TSH levels was observed in females administered 6'-SL sodium salt at 2,500 mg/kg body weight/day and 4,000 mg/kg body weight/day; however, these effects were considered to be fortuitous as the individual values remained within the Historical Control Data range and no microscopic changes in the thyroid gland were noted. As such, the observed changes in the TSH levels are not of any safety concern.

Question 6. Inbiose discusses in Section 6.3.1.5 on page 42 of the notice the results of their unpublished studies with respect to toxicokinetics of 6'-SL oral exposure in rats. Please provide a short narrative describing why the results you reported would not be considered counter to your GRAS conclusion.

6'-sialyllactose is a nondigestible oligosaccharide found in human milk. A brief summary of the available information related to the ADME properties of HMOs, including 6'-SL, was provided in

Section 6.2 of the GRAS notice. Once ingested, it is understood that a small fraction of 6'-SL is quickly absorbed into circulation (Vazquez *et al*., 2017). This behavior was confirmed in the referenced toxicokinetic studies discussed in Section 6.3.1.5, wherein the primary objective was to evaluate the achieved systemic exposure to Inbiose's 6'-SL, in the form of sodium salt, and its relationship to dose level throughout the study. Within these toxicokinetic studies, it was reported that small quantities of orally administered 6'-SL was systemically available in all animals reaching the maximum plasma concentration 1 hour post-administration.

Urinary excretion of 6'-SL was evaluated *via* the measurement of 6'-SL in urine samples collected over a 24-hour period following oral administration of 6'-SL sodium salt on PND 87 or 89. Results from the toxicokinetic studies indicate that the amount of 6'-SL recovered in the urine of rats, collected over a period of 24 hours following dose administration, was below 1% of the daily exposure (calculated as percentage of the administered doses). These results are comparable to the excretion profile of HMOs from breastfed infants, for which only minimal levels of ingested HMOs are detected unchanged in the urine (*i.e.*, 1 to 3% of the total HMO fraction) (Rudloff and Kunz, 2012; Rudloff *et al*., 2012; Goehring et al., 2014). Therefore, Inbiose's 6'-SL product, when added to infant formula, is expected to have similar toxicokinetic profile to 6'-SL naturally present in human breast milk. Thus, the applicant considers that the toxicokinetic results observed in rats do not counter the overall GRAS conclusion.

References:

Goehring KC, Kennedy AD, Prieto PA, Buck RH (2014). Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PLoS ONE 9(7):e101692 [11pp]. DOI:101610.101371/journal.pone.0101692. Cited In: Advanced Protein Technologies, Corp., 2020 [GRN 932, Pt. 1].

Rudloff S, Kunz C (2012). Milk oligosaccharides and metabolism in infants. Adv Nutr 3(3, Suppl.):398S-405S. DOI:10.3945/an.111.001594.

Rudloff S, Pohlentz G, Borsch C, Lentze MJ, Kunz C (2012). Urinary excretion of *in vivo* ^{13}C-labelled milk oligosaccharides in breastfed infants. Br J Nutr 107(7):957-963. DOI:10.1017/S0007114511004016.

Question 7. *On page 24 of the notice, Inbiose states that an updated comprehensive search of the publicly available scientific literature was conducted through March 2021. Please provide an updated literature search that explains how any newly found publicly available information still supports your GRAS conclusion. We note that, although Inbiose states on page 47 that no 6'-SL study in adult humans has been identified, we found a study that may be relevant to your safety assessment (Kim et al., 2022 Reg. Tox. Pharm. 129: 105110).*

To address this question, Inbiose has conducted an updated literature search through January 2023 to identify any new publicly available data pertaining to the safety of 6'-SL that have been published since the original literature search was conducted in March 2021. No new data were identified in the updated search of the published literature that could be perceived as counter to Inbiose's 6'-SL GRAS conclusion; however, several new clinical studies were identified in support of the GRAS conclusion. While the results from these studies are not counter to the GRAS conclusion, these

studies are summarized in Table 1, below, for completeness. Briefly, 6'-SL (alone or in combination with other HMOs) was not observed to elicit adverse effects in humans. Results from these recently published studies corroborate the conclusion that 6'-SL is safe and well-tolerated in infants and adults when provided at levels consistent with the proposed uses of Inbiose's 6'-SL described in the GRAS Notice. Inbiose therefore maintains that this 6'-SL ingredient is GRAS, based on scientific procedures, for use in non-exempt term infant formula and specified conventional food and beverage products, as described in the GRAS Notice.

Table 1 -- Summaries of Newly Identified Clinical Trials Conducted with 6'-SL

Randomized, controlled, parallel-group clinical study	Term, healthy infants ≤14 days of age	112 ± 3 days	Infant formula mixed with 5HMO-Mix (2.99 g/L 2'-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3'-SL, and 0.28 g/L 6'-SL) (N = 86) Control formula without HMOs (N = 91) Placebo: Breast milk (N = 88)	The results demonstrated that 5 HMO-Mix at 5.75 g/L in infant formula was safe and well tolerated by healthy term infants during the first months of life.	Parschat *et al.* (2021)
Randomized, triple-blind, placebo-controlled study	Healthy adults aged 20 years or older	12 weeks	Test group: 3 g of 6'-SL powder orally administrated twice a day after the morning and evening meals at intervals of 12h (N = 30) Placebo: 3 g of maltodextrin power orally administrated twice a day after the morning and evening meals at intervals of 12h (N = 30)	There were no major serious or life-threatening adverse reactions. There were no clinically significant differences among the baseline, 6th, and 12th week clinical chemistry tests, such as aspartate aminotransferase, alanine aminotransferase, and creatinine.	Kim *et al*. (2022)

Table 1 -- Summaries of Newly Identified Clinical Trials Conducted with 6'-SL

Randomized, double-blind, controlled parallel feeding growth trial	Healthy term infants (gestational age 37–42 weeks) between 0 and 14 days of age with a birth weight ≥ 2,490 g.	Time of enrolment at ≤14 Days (D) of age until D 119 or up to D 183	Control milk-based formula (CF; n = 129); experimental formula (EF; N = 130) containing five HMOs (5.75 g/L; 2'-FL (3.0 g/L), 3-FL (0.8 g/L), LNT (1.5 g/L), 3'-SL (0.2 g/L) and 6'-SL (0.3 g/L); reference group: human milk (HM; N = 104)	No significant differences among the three groups for weight gain per day and gains in weight and length (p ≥ 0.05) from D 14 to D 119. Color of stool, its consistency and frequency per day were more similar between EF and HM groups. Serious and non-serious adverse events were not different among groups. The results indicated that EF containing five HMOs was safe and well-tolerated and supported age-appropriate growth.	Lasekan *et al*. (2022)

Table 1 -- Summaries of Newly Identified Clinical Trials Conducted with 6'-SL

Randomized, controlled, double-blind trial	Healthy full-term infants (7–21 days old)	Time of enrolment of age until up to 6 months	Test group 1 (TG1) fed standard infants formula with a concentration of 1.5 g/L of the five-HMO blend: 0.87, 0.10, 0.29, 0.11 and 0.14 g/L for 2'-FL, DFL, LNT, 3'-SL and 6'-SL, respectively. Test group 2 (TG2) fed standard IF with a concentration of 2.5 g/L of the five-HMO blend: 1.45, 0.14, 0.48, 0.18 and 0.24 g/L for 2'-FL, DFL, LNT, 3'-SL and 6'-SL, respectively. Control group (CG): standard cow's milk-based infant formula Placebo: standard IF without HMOs (HMG)	Relative abundance of *Bifidobacterium longum* subsp. *infantis (B. infantis)* was higher in TGs vs. CG. At both post-baseline visits, toxigenic *Clostridioides difficile* abundance was 75–85% lower in TGs vs. CG ($P < 0.05$) and comparable with HMG. At 3 months, TGs (vs. CG) had higher secretory immunoglobulin A (sIgA) and lower alpha-1-antitrypsin ($P < 0.05$).	Bosheva *et al*. (2022)

2'-FL = 2'-fucosyllactose; 3'-FL = 3'-fucosyllactose; 3´-SL= 3´-sialyllactose; 6´-SL= 6´-sialyllactose; CG = control group; D = day(s); DFL= difucosyllactose; EF = experimental formula; h = hour(s); HMG = human milk group; LNT = lacto-N-tetraose; N = number of participants; TG = test group.

Parschat *et al.* (2021) evaluated the effects of infant formula supplemented with 5 HMOs mixture (5.75 g/L total, comprising 52% 2'-FL, 13% 3'-FL, 26% LNT, 4% 3'-SL, and 5% 6'-SL) in healthy term infants. The increase of mean daily body weight and changes in anthropometric parameters, such as weight, length, and head circumference, were recorded over a 4-month period. The safety was measured *via* occurrence of adverse events, while the tolerability and behavioral parameters were measured *via* stool frequency and consistency, gurgitation, vomiting, flatulence, fussiness, crying, and awakening at night. The infants were allocated to test (N = 86) and control (N = 91) group and received infant formula with 5HMO-Mix and infant formula without 5HMO-Mix, respectively. In addition, a reference breast-fed infant group was included (N = 88). No differences in weight, length, or head circumference gain were observed between the two formula groups. The frequency of AEs in the two formula groups were similar, which was slightly but not significantly higher than that for the breast-fed group. The authors showed that the mixture of 5 HMOs had positive effect on normal infant growth and was safe and well tolerated for use in healthy term infants.

In a single-center, randomized, triple-blind, controlled clinical study, the safety of 6'-SL for human use was investigated by Kim *et al.* (2022). 60 healthy adults were recruited and randomly assigned to test group (6g orally per day divided in two doses after the morning and evening meals at intervals of 12h for 12 weeks) and placebo group (6g of maltodextrin power at same schedule as test group). Clinical chemistry tests consisted of total protein, albumin, total cholesterol, triglyceride, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γ-GTP), total bilirubin, glucose, BUN, and serum creatinine were conducted at the sixth and twelfth weeks after the start of intake. No serious adverse reactions, such as life-threatening complications requiring hospitalization, causing disability, or causing deformity were observed during the use of 6'-SL. Some minor adverse reactions mainly related with the gastrointestinal symptoms such as abdominal discomfort and diarrhea, were noted in both test and placebo groups. Overall, no significant differences in the number of adverse drug reaction were shown. There were also no statistically significant differences in the clinical laboratory test results between groups. The authors concluded that daily dose of 6'-SL up to 6 g is safe in adults and can be used for 12 weeks without major adverse reactions.

Lasekan *et al.* (2022) conducted a randomized, controlled, multicenter, double-blind, parallel feeding growth and tolerance study to investigate the growth and gastrointestinal tolerance of milk-based infant formula supplemented with 5 HMOs in healthy term babies. The 5 HMO blend content used in this study was exactly the same as used by Parschat *et al.* (2021), *i.e.*, 3.0 g/L of 2'-FL, 0.8 g/L of 3'-FL, 1.5 g/L of LNT, 0.2 g/L of 3'-SL, and 0.3 g/L of 6'-SL. Infants were randomized to receive either a control (N = 129) or an experimental formula with blend of 5 HMOs (N = 130) through approximately 4 months of age. The breastfed infants (N = 101) were included as a reference group. Weight, length, head circumference (HC), mean rank stool consistency (MRSC) number of stools per day and a percentage of feedings with spit-up/vomit associated with feeding were measured from day (D) 14 to D119. No differences were observed among the three groups for weight gain per day from D14 to D119 days ($p \geq 0.337$). Infants fed with experimental formula had more soft, frequent and yellow stools and were similar to the reference group. There were no differences between serious and non-serious adverse events among three groups. The blend of 5 HMOs was concluded to be safe and well-tolerated as well as supportive of normal growth. These results were in line with data published by Parschat *et al.* (2021).

In a randomized, controlled, double-blind trial, Bosheva *et al.* (2022) investigated the gut maturation effects (microbiota, metabolites, and selected maturation markers) of an infant formula containing a

specific blend of five HMOs. Healthy full-term infants were assigned to control group (CG) fed a standard IF without HMOs, test group 1 (TG1) and test group 2 (TG2) fed with the same standard IF containing the five-HMO blend at a concentration of 1.5 g/L and 2.5 g/L, respectively. A non-randomized human milk-fed infants (HMG) served as reference group. Fecal samples collected at baseline, age 3 and 6 months, were analyzed for microbiome (shotgun metagenomics), pH and organic acids, as well as the biomarkers (immunoglobulin A (sIgA), calprotectin and alpha-1-antitrypsin). Higher bifidobacterial and lower toxigenic *C. difficile* abundance were observed in the TGs vs. CG. Early life intestinal immune response was improved as indicated by the higher fecal sIgA concentration in the TGs vs. CG. The authors concluded that the infant formula contained specific HMO blend was able to support the development of the intestinal immune system and shaped the gut microbiota directionally toward that of breastfed infants.

References

Bosheva, M., Tokodi, I., Krasnow, A., Pedersen, H. K., Lukjancenko, O., Eklund, A. C., Grathwohl, D., Sprenger, N., Berger, B., & Cercamondi, C. I. (2022). Infant Formula With a Specific Blend of Five Human Milk Oligosaccharides Drives the Gut Microbiota Development and Improves Gut Maturation Markers: A Randomized Controlled Trial. *Frontiers in Nutrition*, *9*. https://doi.org/10.3389/fnut.2022.920362

Kim, J. H., Yong, S. Y., Kim, S. H., Baek, A., Go, T. H., & Kang, D. R. (2022). Randomized, triple-blind, placebo-controlled study to evaluate the safety of 6'-Sialyllactose in healthy adults. *Regulatory Toxicology and Pharmacology*, *129*. https://doi.org/10.1016/j.yrtph.2021.105110

Lasekan, J., Choe, Y., Dvoretskiy, S., Devitt, A., Zhang, S., Mackey, A., Wulf, K., Buck, R., Steele, C., Johnson, M., & Baggs, G. (2022). Growth and Gastrointestinal Tolerance in Healthy Term Infants Fed Milk-Based Infant Formula Supplemented with Five Human Milk Oligosaccharides (HMOs): A Randomized Multicenter Trial. *Nutrients*, *14*(13), 2625. https://doi.org/10.3390/nu14132625

Parschat, K., Melsaether, C., Jäpelt, K. R., & Jennewein, S. (2021). Clinical Evaluation of 16-Week Supplementation with Tolerability , Safety , and Effect on Growth. *Nutrients*, *13*, 2871.