Glycom A/S Kogle Allé 4 2970 Hørsholm, Denmark

21 November 2018

833

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

Dear Dr. Gaynor:

Re: GRAS Notice for lacto-*N*-tetraose (LNT)

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Glycom A/S [Kogle Allé 4 2970 Hørsholm, Denmark], as the notifier, is submitting one hard copy and one electronic copy (on CD), of all data and information supporting the company's conclusion that lacto-*N*-tetraose (LNT) produced with an *E. coli* K12 (DH1)-derived strain, is GRAS on the basis of scientific procedures, for use in non-exempt term infant formula and specified conventional food and beverage products across multiple categories; these food uses of LNT are therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for Glycom's GRAS conclusion, as well as a consensus opinion of an independent panel of experts, also are enclosed for review by the agency.

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

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

Sincerely,

[redacted]

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

GRAS NOTICE FOR LACTO-*N*-TETRAOSE (LNT)

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

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

DATE:
21 November 2018

GRAS Notice for Lacto-*N*-Tetraose (LNT)

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GRAS Notice for Lacto-*N*-Tetraose (LNT)

Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through 170.285, Glycom A/S (Glycom) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that lacto-*N*-tetraose (LNT), as manufactured by Glycom, is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Glycom's view that the notified substance is Generally Recognized as Safe (GRAS) under the conditions of its intended use described in Section 1.3 below. In addition, as a responsible official of Glycom, the undersigned hereby certifies that all data and information presented in this notice represents a complete, representative, and balanced submission, and considered all unfavorable as well as favorable information known to Glycom and pertinent to the evaluation of the safety and GRAS status of LNT as a food ingredient for addition to non-exempt term infant formula and various conventional food products, as described herein.

Signed,

21.11.2018

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

Date

1.1 Name and Address of Notifier

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

1.2 Common Name of Notified Substance

Lacto-*N*-tetraose; LNT

1.3 Conditions of Use

LNT is intended to be added as a food ingredient to foods targeted to infants and young children, including non-exempt term infant formula, as well as uses in specific conventional food and beverage products used by the general population (Table 1.3-1). Food uses of LNT in infant formula (*i.e.*, infants up to 12 months) will provide LNT at a use-level of 0.8 g/L, follow-on formula at a use-level of 0.6 g/L, infant-specific foods and foods for young children at a use-level of 0.6 g/L in ready-to-drink and reconstituted products, and up to 5 g/kg for products other than beverages (*e.g.*, baby foods). LNT is also intended for use in food and

beverages targeted towards the general U.S. population (up to 1.0 g/L or 10 g/kg), and foods for special dietary use (*e.g.*, meal replacement bars) at levels up to 2.0 g/L or 20 g/kg. The maximum use-levels are proposed on the basis of providing similar levels of LNT on a body weight basis as those consumed by breast-fed infants (see Section 3.1.3).

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

Food Category	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use-Level (g/RACC)	Proposed Maximum Use-Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction[b]	240 mL	0.48	2.0
	Sports and Isotonic Drinks, Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	360 mL	0.36	1.0
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.08	0.8
	Toddler Formulas	100 mL[c]	0.06	0.6
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.04 to 0.85	5.0
	Other Drinks for Young Children	120 mL	0.07	0.6
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	0.8	20
	Cereal and Granola Bars	40 g	0.4	10
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk*	240 mL	0.24	1.0
Milk Products	Buttermilk*	240 mL	0.24	1.0
	Flavored Milk	240 mL	0.24	1.0
	Milk-Based Meal Replacement Beverages, for Weight Reduction[b]	240 mL	0.48	2.0
	Yogurt*	170 g	1.7	10

LNT = Lacto-*N*-tetraose; CFR = Code of Federal Regulations; RACC = Reference Amounts Customarily Consumed; U.S. = United States.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2018aa). 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.

*LNT is intended for use in unstandardized products when standards of identity do not permit its addition.

1.4 Basis for GRAS

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

1.5 Availability of Information

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

Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

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

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

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

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

2.1 Identity

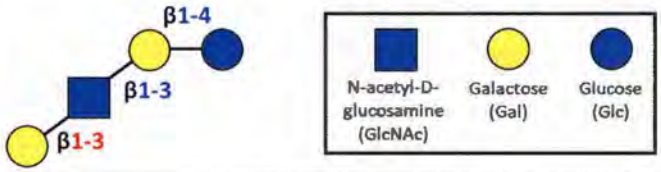
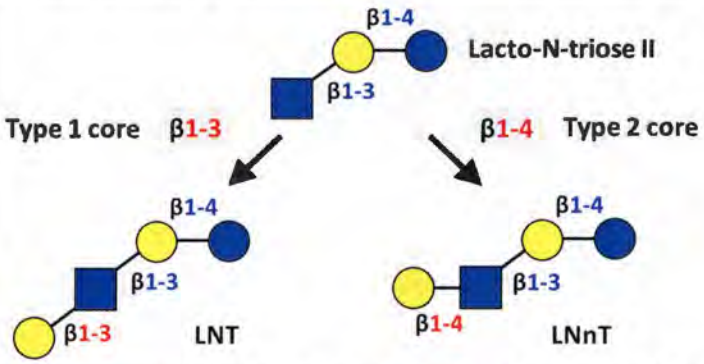
LNT is predominantly a single purified ingredient, but also contains small quantities of lactose and lacto-*N*-triose II [all 3 are collectively called human-identical milk saccharides (HiMS)[1]]. The final product also contains minor amounts of other related and fully characterised carbohydrates, which are produced during the fermentation process. LNT is a tetrasaccharide that is derived from lactose by subsequent addition of *N*-acetylglucosamine (GlcNAc) and galactose in linkage-specific manner. Further parameters of the identity of LNT, such as chemical names, molecular structure, molecular weight and the structural relationship between the 3 HiMS are summarized in Table 2.1-1 below.

As shown in Table 2.1-1, LNT and LNnT share a highly similar chemical structure, with the notable difference that LNT contains a terminal Gal-β(1-3)-GlcNAc linkage (type 1), while LNnT contains a terminal Gal-ß(1-4)-GlcNAc linkage (type 2). Lacto-*N*-triose II is a biochemical precursor of the naturally occurring HMOs LNT and LNnT, and, is therefore similarly present at low levels as a by-product of the manufacturing process in the ingredients.

The LNT produced by microbial fermentation is chemically and structurally identical to LNT that is naturally present in human breast milk, as confirmed by 1H- and 2D-nuclear magnetic resonance (NMR)-spectroscopy and mass spectrometry.

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

Table 2.1-1 Description of Identity of LNT

Product Name	Lacto-*N*-tetraose
Abbreviations	LNT
IUPAC Name	β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
IUPAC Abbreviation (extended)	β-D-Gal*p*-(1-3)-β-D-GlcNAc*p*-(1-3)-β-D-Gal*p*-(1-4)-D-Glc
IUPAC Abbreviation (condensed)	Gal-(β1-3)-GlcNAc-(β1-3)-Gal-(β1-4)-Glc
Molecular Structure	
Symbol Nomenclature	
Molecular Formula	$C_{26}H_{45}O_{21}$
Molecular Mass (weight)	707.63
CAS Number	14116-68-8
CAS Name	D-Glucose, O-β-D-galactopyranosyl-(1→3)-O-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-
The structural relationship between LNT, LNnT and lacto-*N*-triose II	
	

CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry; LNnT = Lacto-*N*-neotetraose; LNT = Lacto-*N*-tetraose.

2.2 Manufacturing

2.2.1 Description of the Production Microorganism

2.2.1.1 Parental (Host) Strain

The genotypic characteristics of the parental/recipient microorganism, *Escherichia coli* K-12 DH1, are presented in Table 2.2.1.1-1. The genome of *E. coli* K-12 has been sequenced and bioinformatic

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

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

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

2.2.1.2 Production Strain

The fermentation organism for LNT is *E. coli* (K-12 DH1 MDO) MP813, a stable and reliable production strain that provides high titres of LNT. The strain has been deposited in the DSMZ in Braunschweig, Germany, under DSM No. 32776. Strain MP813 was developed by modification of Glycom's MDO platform strain (see below for further details). As discussed previously, the MDO platform strain includes several genetic modifications, primarily gene deletions of carbohydrate utilization enzymes, which optimize the productivity of the production organism by attenuating unwanted carbohydrate catabolism pathways. Details of the modification steps used for construction of strain MDO has been discussed previously in GRN 650 and is hereby incorporated by reference to Section II.B.1.2 of GRN 650 (U.S. FDA, 2016a).

[2] www.dsmz.de

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

The biochemical pathway by which the production strain generates LNT using D-lactose and D-glucose[4] as a substrate and carbon source, respectively, is shown in Figure 2.2.1.2-1.The MDO platform strain was genetically modified to biosynthesize LNT in a two-step process: 1) conversion of lactose to lacto-*N*-triose II (step A); and 2) conversion of lacto-*N*-triose II to LNT (step B).

To facilitate the conversion of lactose to lacto-*N*-triose II, the MDO platform strain was genetically modified to genomically and episomally overexpress (4 genomic and 1 episomal copy) the gene encoding for β-1,3-*N*-acetylglucosaminyl-transferase, which is involved in the transfer of a GlcNAc unit from the intracellular UDP-GlcNAc pool to the 3'-position of the galactose residue of lactose in a β-stereospecific manner to form the trisaccharide lacto-*N*-triose II. Multiple copies were inserted at multiple sites to allow for high intracellular levels of lacto-*N*-triose II. Next, to facilitate the conversion of lacto-*N*-triose II to LNT, the MDO platform strain was genetically modified to episomally express the gene encoding for β-1,3-galactosyl-transferase, which further converts lacto-*N*-triose II to the tetrasaccharide LNT. The wild-type (non-codon-optimized) coding sequence of the β-1,3-*N*-acetylglucosaminyltransferase gene (originating from *Neisseria meningitidis*) and the wild-type (non-codon-optimized) coding sequence of the a β-1,3-galactosyltransferase enzyme (originating from *Helicobacter pylori*) were cloned as an operon-like structure in a high-copy number plasmid (pBS) and expressed in tandem under the control of a single wild-type P*lac* promoter that is native to *E. coli*. In this construct[5], two distinct Shine-Dalgarno sequences were included in front of each heterologous gene. Specifically, the construct corresponds to an expression cassette that contains the wild-type P*lac* sequence followed by a Shine-Dalgarno sequence, the wild-type β-1,3-galactosyltransferase enzyme coding sequence, 33 random nucleotide bases, another Shine-Dalgarno sequence, and the wild-type β-1,3-*N*-acetylglucosaminyltransferase enzyme coding sequence.

The gene encoding for quinolinate phosphoribosyl-transferase involved in NAD biosynthesis, a co-factor that is essential for growth in minimal medium, was deleted from the genome of the production organism in a two-step process. This gene is provided to the cells from a plasmid (same plasmid as described above for β-1,3-galactosyltransferase) in order to tightly link cell survival and LNT production with plasmid retention. The final plasmid construct is free of antibiotic markers and enables the production of LNT in high titers.

Although the strain MP813 bears a plasmid, no antibiotic resistance genes are present in the organism and no antibiotics or inducer molecules are used throughout the whole LNT fermentation process.

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

[5] The vector was produced from the pBluescript II KS + vector (pBS_II_KS+), which is a commercial vector developed by Stratagene. It contains the pUC origin of replication, which is derived from the pBR322 pMB1 replicon. Due to the deletion of the *rop* gene, it is maintained at a very high copy number (several hundred copies per cell). It carries the resistance for ampicillin and contains a P*lac* promoter.

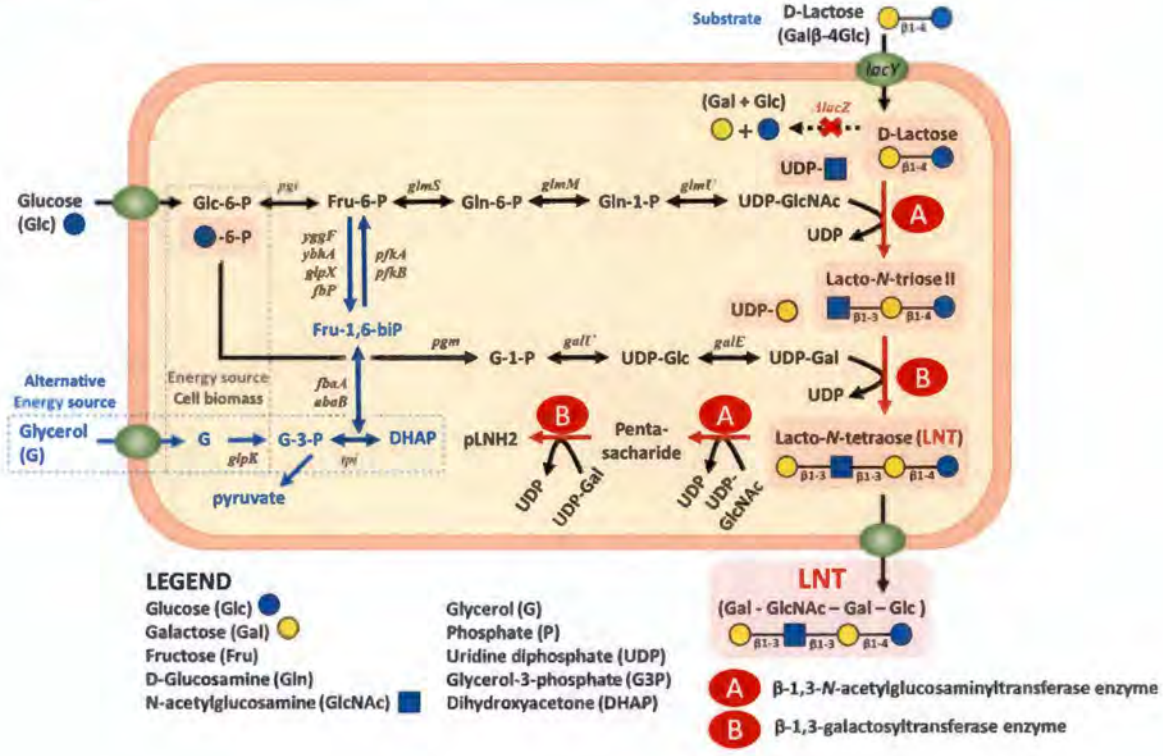


Figure 2.2.1.2-1 Engineered Pathway for LNT Biosynthesis in the Production Strain *Escherichia coli* (K-12 DH1 MDO) MP813

The enzyme identities are well characterized and based on their enzymatic functions it was concluded that the introduced genes would not confer toxicogenic/pathogenic properties to the host organism. The genetic modifications made to the production strains result in the expression of proteins that are involved in the normal carbohydrate processing within their donor microbial (bacterial) sources. These proteins are "carbohydrate-active enzymes" ("CAZy"), a panel of enzymes that can degrade, modify, or create glycosidic bonds, and accordingly are involved in the metabolism of complex carbohydrates[6]. When expressed together in the recipient strains, these proteins work in concert to convert the starting carbohydrates (D-lactose and D-glucose[7]) into oligosaccharides that are identical to those in human breastmilk. In contrast, bacterial protein toxins (exotoxins) are known to mediate their pathogenic effects by disrupting cellular processes through various mechanisms such as proteolysis (e.g., tetanus and botulinum), ADP-ribosylation (e.g., cholera, pertussis, and diphtheria), or membrane disruptions through pore formation (Finlay and Falkow, 1997; Popoff, 2018; Wilson *et al.*, 2002). Indeed, bioinformatic searches conducted using the amino acid sequences of the proteins introduced to the *E. coli* K-12 (DH1) MP813 strain by genetic modification confirmed that there is no relevant homology to known protein toxins or to known allergens. The genetic modifications applied to the platform and production strains were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods.

During manufacture, the production strain remains intact, secretes the LNT extracellularly, and then is entirely removed through a series of purification steps (as described in Section 2.2.2). Therefore, in this process the production strain is used exclusively as a processing aid.

[6] http://www.cazy.org/Welcome-to-the-Carbohydrate-Active.html.
[7] D-Glycerol and D-sucrose are alternative carbon sources to D-glucose.

2.2.2 Description of the Production Process

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

In Stage 1 [upstream processing (USP)], D-lactose and D-glucose are converted to LNT by the adapted cellular metabolism of the LNT production microorganism, which uses D-glucose[8] as an exclusive energy and carbon source and D-lactose as a substrate for LNT biosynthesis. The production microorganism is removed from the fermentation medium at the end of the fermentation process.

In Stage 2 [downstream processing (DSP)], a series of purification, isolation and concentration steps are used to generate the final high-purity LNT product.

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

Table 2.2.2-1 Overview of the Manufacturing Process for the LNT Product

STAGE 1		**Upstream Processing (USP)**
STEPS	1	Media Preparation
	2	Propagation - Working cell bank (WCB)
	3	Seed Fermentation
	4	Fermentation Phases:
	4A	Growth (Batch) Phase
	4B	Feeding (Fed-Batch) Phase
	5	Removal of Microorganism - Ultrafiltration/diafiltration (UF/DF)
STAGE 2		**Downstream Processing (DSP)**
STEPS	6	Purification/Concentration 1 - Nanofiltration or nanofiltration/diafiltration (NF1/DF) membranes
	7	Ion Removal - Ion exchange resin (IEX)
	8	Decolourization - Active charcoal (AC)
	9	Purification/Concentration 2 - Nanofiltration or nanofiltration/diafiltration (NF2/DF) membranes
	10	Drying - Spray/freeze drying
	11	Sampling and Packaging
	12	Quality Control and Batch Release

LNT = lacto-*N*-tetraose.

2.2.3 Quality Control

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

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

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

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

The HACCP plan for both manufacturing stages comprise in-process controls to minimize the amount of impurities to the level technically possible. Glycom's production process (including all processing aids, raw materials, unit operations, and filter aids) and food safety management system comply with the following standards and certifications: Food Safety Systems Certification 22000 (FSSC 22000), ISO 9001, Kosher, Halal, Bisphenol-A free, Phthalate-free, Latex-free, and Allergen-free (except for milk-derived allergens).

The production microorganism is efficiently removed by the ultrafiltration step, which is applied directly after the fermentation. There are several subsequent purification steps involved in the down-stream processing stage to obtain a highly purified LNT, free of the bacterial cells and residual fermentation by-products. The absence of the microorganisms is demonstrated by measuring *Enterobacteriaceae* in the final product according to internationally recognized methods (ISO 21528-1:2004, MSZ ISO 21528-2:2004). The ISO 21528-1:2004 method includes a pre-enrichment step for detection of microorganisms that may require resuscitation before enrichment.

E. coli K-12 is a gram-negative bacterium and these bacteria possess complex glycolipids of high molecular weight, called lipopolysaccharides (LPS), in their cell membrane. LPS elicits an immune response when introduced to the blood stream. Therefore, LPS can cause inflammatory responses when present as contaminants in infusion therapy procedures; LPS is also called endotoxin (not to be confused with protein-type toxins); however, LPS from *E. coli*-K12 lacks O-antigen epitopes that are characteristic of pathogenic strains of *E. coli*. LPS are comparably harmless when ingested, most likely due to a combination of deactivation by stomach acid and a low absorption due to the high molecular weight. Moreover, as LPS is an intrinsic component of the cell membrane of gram-negative bacteria, background exposure of the gastrointestinal tract to LPS from the resident microbiota is high. To control for potential residual endotoxin levels, a strict specification for endotoxins has been set for the ingredient and all batch results confirm high purity in terms of endotoxins.

After fermentation with strain MP813 and purification of LNT, the absence of traces of residual DNA in the product is confirmed by 3 different validated quantitative PCR (qPCR) methods which target short sub-sequences of the acetylglucosaminyltransferase and the galactosyltransferase genes as well as a short sub-sequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. These methods have been validated to detect traces of DNA down to 4 µg/kg (parts per billion). All the qPCR tests were applied to all analyzed batches and the results were below the limit of quantification (LOQ) in all tested batches (see Table 2.3.3.2-1).

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

Specifications for LNT are presented in Table 2.3.1-1. The parameters include the main components of the HiMS mixture, consisting predominantly of LNT (min. 70%), with some levels of lactose (max. 12%) and lacto-*N*-triose II (max. 10%), as well as possible by-products and degradation products[9] such as *para*-lacto-*N*-hexaose-2 (max. 3.5%) and the LNT fructose isomer[10] (max. 1%).

LNT is further specified as a white to off-white powder that is produced by a microbial fermentation process using an *E. coli* K-12 DH1-derived strain. Since lactose and lacto-*N*-triose II are also naturally present in human milk, another quality parameter relevant to the infant nutrition uses has been introduced with 90.0% as the sum of HiMS to ensure a highly consistent product quality in context of that particular proposed use. The LNT content is specified by water-free assay based on high-performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) analysis. The main advantages of the PAD detector used is high sensitivity and selectivity, wide dynamic detection levels, reliable performance with excellent precision and more consistent response over wide range of chemical structures (*e.g.*, no chromophores are required for detection). Upper limits have also been established for microbiological parameters and includes separate specifications for LNT that is used during the wet blending stage of infant formula where heat killing steps are applied prior to dry-blending of heat-sensitive ingredients; infant formula containing LNT will therefore be compliant with the microbial requirements for infant formula as defined under 21 CFR §106.55. These microbial specifications are comparable to limits concluded to be GRAS for the addition of galacto-oligosaccharides to infant formula as described in GRN 620.

All methods of analysis are either internationally-recognised or developed and validated internally by Glycom and confirmed by independent accredited external laboratories [International Laboratory Accreditation Cooperation (ILAC)-accredited laboratory WESSLING Hungary Kft., and Eurofins Medigenomix GmbH (Germany) accredited against ISO/IEC 17025:2005 by Die Deutsche Akkreditierungsstelle GmbH (DakkS)].

Table 2.3.1-1 Specifications for LNT

Definition				
Lacto-*N*-tetraose (LNT) is a purified, white to off-white powder that is produced by a microbial process.				
Source				
A modified strain of *Escherichia cali* K-12 DH1.				
Parameter	**Specification**	**AVE**	**± SD**	**Method**
Appearance	Powder or agglomerates	Complies		ISO 6658:2007
Colour	White to off white	Complies		ISO 6658:2007
Identification (LNT)	RT of standard ± 3%	Complies		Glycom method HPLC-703-7C7-001
Assay (water free) HiMS[a]	Not less than 90.0 w/w %	93	± 1	Glycom method HPLC-703-7C7-001 and -006
Assay (water free) – LNT	Not less than 70.0%	78	± 2	Glycom method HPLC-703-7C7-001
D-Lactose	Not more than 12.0 w/w %	7.6	± 1.5	Glycom method HPLC-703-7C7-006
Lacto-*N*-triose II	Not more than 10.0 w/w %	6.4	± 1.0	Glycom method HPLC-703-7C7-006

[9] It should be noted that lactose and lacto-*N*-triose II are also principal degradation products of LNT.
[10] "LNT fructose isomer" denotes an isomer of LNT where the terminal glucose sugar isomerised to a fructose sugar.

Table 2.3.1-1 Specifications for LNT

Para-LNH2	Not more than 3.5 w/w %	2.8	± 0.3	Glycom method HPLC-703-7C7-006
LNT fructose isomer	Not more than 1.0 w/w %	0.7	± 0.1	Glycom method HPLC-703-7C7-001
Sum of other carbohydrates	Not more than 5.0 w/w %	1.3	± 0.1	Glycom method HPLC-703-7C7-001 and -006
pH (20°C, 5% solution)	4.0 to 6.0	4.4	± 0.2	Ph. Eur. 9.2 2.2.3 (07/2016:20203)
Water	Not more than 6.0 w/w %	1.9	± 0.5	Glycom method KF-001
Ash, sulphated	Not more than 0.5 w/w %	0.10	± 0.03	Ph. Eur. 9.2 2.4.14 (04/2010:20414)
Residual protein by Bradford assay	Not more than 0.01 w/w %	< 0.0017		Glycom method UV-001
Microbiological Parameters[b]				
Aerobic mesophilic total plate count	Not more than 500 CFU/g	< 10		ISO 4833-1:2014
Enterobacteriaceae	Absent in 10 g	Complies		ISO 21528-1:2004, ISO 21528-2:2004
Salmonella spp.	Absent in 25 g	Complies		ISO 6579:2006
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Complies		ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	Complies		ISO 11290-1:1996/A1:2005
Bacillus cereus	Not more than 50 CFU/g	< 10		ISO 7932:2005
Yeasts	Not more than 10 CFU/g	< 10		ISO 7954:1999
Moulds	Not more than 10 CFU/g	< 10		ISO 7954:1999
Residual endotoxins	Not more than 10 EU/mg	0.02	± 0.02	Ph. Eur. 2.6.14

AVE = average; CFU = colony-forming units; EU = endotoxin units; Ph. Eur. = European Pharmacopoeia; HiMS = Human-identical milk saccharides (LNT + Lactose + Lacto-*N*-triose II); HPAEC = high-performance anion exchange chromatography; IC = ion chromatography; ISO = International Organization for Standardization; KF: Karl-Fischer; MPN = most probable number; RT= retention time.

[a]HiMS = Sum of LNT + Lactose + Lacto-*N*-triose II.

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

2.3.2 Product Analyses

2.3.2.1 Main products and Other Carbohydrates

LNT manufactured by Glycom can be described as white to off-white amorphous powder or agglomerate. Amorphous powders do not possess defined melting points. LNT is readily soluble in aqueous solutions (max. 400 mg/mL, 25°C), with poor solubility in any organic solvents. The summary of batch results corresponding to selected physicochemical properties of LNT is presented in Table 2.3.2.1-1.

Table 2.3.2.1-1 Batch Results for Selected Physicochemical Properties of LNT Product

Parameters	Manufacturing Batch Numbers:							
	CPN4215 1000216 FD	CPN4215 1000316 FD	CPN4215 1000416 FD	CPN4215 1000516 FD	CPN4215 1000616 FD	AVE	±	SD
Appearance	Powder or agglomerates							
Colour	White to off white							
pH (20°C, 5% solution)	4.6	4.0	4.3	4.5	4.4	4.4	±	0.2

AVE = average; LNT = lacto-*N*-tetraose; SD = standard deviation.

The analytical results of 5 independent production batches of LNT together with averaged values (AVE) and standard deviations (SD) are provided in the Table 2.3.2.1-2 below. Results of the HPAEC-PAD analyses demonstrate that LNT, contributes on average to ~78% by weight of the simple mixture (as shown in Table 2.3.2.1-2). The collective HiMS fraction (LNT, lactose and lacto-*N*-triose II) of the product comprises on average 93% of the total batch weight. The small remaining portion of the product consists mainly of other carbohydrate-type compounds structurally related to LNT, *e.g.*, para-lacto-*N*-hexaose-2 (para-LNH2) or the "LNT fructose isomer" (the chemical identity of the latter is explained below). The water-free total specified carbohydrates fraction of the LNT product adds up to 98% of the final batch weight.

Table 2.3.2.1-2 Batch Results for Fermentation Metabolites and Other Carbohydrates By-Products for LNT

Parameters	Manufacturing Batch Numbers:							
	CPN4215 1000216 FD	CPN4215 1000316 FD	CPN4215 1000416 FD	CPN4215 1000516 FD	CPN4215 1000616 FD	AVE	±	SD
Assay (water free) HiMS [%]	94.7	92.4	92.2	92.0	92.0	**92.7**	±	**1.2**
Assay (water free) – LNT [%]	80.1	80.2	77.0	76.2	78.9	**78.5**	±	**1.8**
D-Lactose [%]	6.27	6.28	8.94	9.33	7.18	**7.6**	±	**1.5**
Lacto-*N*-triose II [%]	8.15	6.28	5.95	6.24	5.55	**6.4**	±	**1.0**
Para-LNH2 [%]	2.38	2.85	2.48	2.93	3.21	**2.8**	±	**0.3**
LNT fructose isomer [%]	0.53	0.69	0.60	0.73	0.71	**0.7**	±	**0.1**
Sum of other carbohydrates [%]	1.1	1.3	1.4	1.4	1.2	**1.3**	±	**0.1**
Total specified carbohydrates (water free) [%]	98.8	97.9	96.7	97.1	97.2	**97.6**	±	**0.8**

AVE = average; HiMS = human-identical milk saccharides; LNT = lacto-*N*-tetraose; para-LNH2 = para-lacto-*N*-hexaose-2; SD = standard deviation.
[a] LoR = 0.03% (w/w).

Lactose is a natural component of human breast milk (*i.e.*, the most abundant single molecule of milk) and the resulting exposure from its level as component of the LNT product is insignificant compared to the exposure from its naturally occurring level in human breast milk.

Lacto-*N*-triose II is a metabolic intermediate of LNT (and LNnT) biosynthesis and one of the products of LNT (or LNnT) hydrolysis (Kuhn *et al.*, 1956); lacto-*N*-triose II therefore is also naturally present in breast milk (Dabrowski *et al.*, 1983; Hosomi and Takeya, 1989; Miwa *et al.*, 2010). Concentrations of lacto-*N*-triose II in human milk have not been reported; however, lacto-*N*-triose II is will be formed as a metabolite of LNnT and LNT microbial metabolism in the human gut (Miwa *et al.*, 2010; Honda *et al.*, 2013; Bidard *et al.*, 2016; Thongaram *et al.*, 2017), and is therefore assumed to occur at significantly higher relative levels in the gut than in breast milk. Lacto-*N*-triose II is also a component of LNnT produced by fermentation (with a specification of up to 3 %), which has GRAS status for a number of food applications, including infant formula, in the United States (*i.e.*, GRN 659).

Other carbohydrate impurities may be detected in the LNT ingredient; however, these compounds are either HiMOs themselves or fall into the general structural patterns observed in HMOs. Such carbohydrates that may form during the fermentation process include para-lacto-*N*-hexaose-2 and "LNT fructose isomer".

The hexasaccharide, para-lacto-*N*-neohexaose-2 (para-LNH2), is an isomer/analogue of para-lacto-*N*-hexaose naturally present in human milk, firstly isolated and described in 1977 by Yamashita *et al.* (1977). The "LNT fructose isomer" is an isomerisation product of LNT where the terminal glucose moiety has isomerised into a fructose unit. Such isomerisation of carbohydrates containing glucose at the reducing end (*e.g.*, glucose, lactose) is also known as the Lobry de Bruyn–van Ekenstein transformation (Angyal, 2001; Wang, 2010). This type of isomerisation is pH- and temperature-dependent and has been commonly reported for the closely related conversion of D-lactose into D-lactulose during heat treatment [*i.e.*, ultra-high temperature (UHT) processing and pasteurisation] of milk, including human donor milk (Beach and Menzies, 1983; Schuster-Wolff-Bühring *et al.*, 2010; Gómez de Segura *et al.*, 2012). Although the presence of the isomerisation product of LNT has not been evaluated in heat-treated human donor milk, D-lactulose has been detected at significant proportions of D-lactose (Gómez de Segura *et al.*, 2012). Also different infant formulas have been reported to contain lactulose at relative levels between 1 and 7% of their lactose content, and absolute levels up to 13.7 mmol/L (Beach and Menzies, 1983). Therefore, it can be reasonably assumed that the LNT fructose isomer is present at comparable ratios to that of heat-treated human milk and can therefore be equally regarded to have a history of safe consumption.

The results presented here demonstrate that the final LNT ingredient complies with the specification (Section 2.3.1). All analyses were performed using internationally-recognised methods or newly developed and validated analytical protocols at Glycom's Research & Development department and confirmed by accredited laboratories to cover a wide range of possible compounds in the final product, and to assure the highest possible precision of measurements and identification of unknown impurities.

2.3.2.2 Non-carbohydrate Residues

All batches were tested for residual water and sulphated ash, which are minor contributors in the composition of LNT (see Table 2.3.2.2-1).

Table 2.3.2.2-1 Batch Results for Water and Sulphated Ash Content of LNT Product

Parameter	Manufacturing Batch Numbers:							
	CPN4215 1000216 FD	CPN4215 1000316 FD	CPN4215 1000416 FD	CPN4215 1000516 FD	CPN4215 1000616 FD	AVE	±	SD
Water [%]	1.5	1.9	2.0	1.6	2.7	**1.9**	±	**0.5**
Ash, sulphated [%]	0.15	0.11	0.08	0.07	0.09	**0.10**	±	**0.03**

AVE = average; LNT = lacto-*N*-tetraose; SD = standard deviation.

2.3.2.3 Microbial Contaminants

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

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

Table 2.3.2.3-1 Batch Results for Microbiological Analysis of LNT

Microbiological Parameters	Manufacturing Batch Numbers: CPN4215 1000216 FD	CPN4215 1000316 FD	CPN4215 1000416 FD	CPN4215 1000516 FD	CPN4215 1000616 FD	AVE ± SD
Aerobic mesophilic total plate count [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**
Enterobacteriaceae	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	**Absent in 10 g**
Salmonella spp.	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	**Absent in 25 g**
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	**Absent in 10 g**
Listeria monocytogenes	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	**Absent in 25 g**
Bacillus cereus [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**
Yeasts [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**
Moulds [CFU/g]	< 10	< 10	< 10	< 10	< 10	**< 10**

AVE = average; CFU = colony-forming units; EU = endotoxin units; LNT = lacto-*N*-tetraose; SD = standard deviation.

2.3.3 Manufacturing By-Products, Impurities and Contaminants

Quality Control measures include testing for carbohydrate-type metabolites and potential residual compounds and trace elements. These include analyses for amino acids, biogenic amines, microbial endotoxins, residual proteins, trace elements and the presence/absence of genes characteristic for the production microorganism. Those that have been confirmed as absent are not proposed for addition to the product specifications.

2.3.3.1 Amino Acids and Biogenic Amines

LNT is secreted into the fermentation broth and no disruption of the production microorganism is required during manufacture. As a precautionary measure, production batches have been analyzed for secondary metabolites and cellular components that may potentially originate from the fermentation medium. Results of analyses of the ingredient for biogenic amines (*e.g.*, histamine, tyramine, spermidine, cadaverine, and putrescine), and amino acids and their metabolites (*e.g.*, glutamic acid and gamma-aminobutyric acid) did not identify detectable levels of these contaminants in any of the manufacturing batches of the finished product (data not shown). Therefore, these compounds do not contribute to the overall compositional data of the LNT final product.

2.3.3.2 Microbial Endotoxins and Residual Proteins

The parental strain, *E. coli* K-12, is a gram-negative bacterium which possess complex glycolipids of high molecular weight in their cell walls, called either LPS or endotoxins (not to be confused with protein-type toxins). Internal specifications for endotoxin have been established [max. 10 endotoxin units (EU)/mg] as an additional quality control point to ensure that any microbial endotoxins are efficiently removed and/or not introduced during the production process. The endotoxin content in the LNT produced by fermentation was assayed using the *Limulus* amoebocyte lysate kinetic chromogenic assay. A sensitive for residual protein based upon a modification of the Bradford assay has been developed by Glycom. Because batch analyses of LNT demonstrated extremely low endotoxin and residual protein concentrations, they were not considered as compositional or safety-related data of the LNT final product (Table 2.3.3.2-1). However, the presence of

residual endotoxins and protein are monitored during routine batch release as an element of HACCP that would allow to identify process deviations in a sensitive manner (see Table 2.3.1-1).

Table 2.3.3.2-1 Batch Results for Microbial Endotoxins and Residual Proteins in LNT Product

Parameters	Manufacturing Batch Numbers:							
	CPN4215 1000216 FD	CPN4215 1000316 FD	CPN4215 1000416 FD	CPN4215 1000516 FD	CPN4215 1000616 FD	AVE	±	SD
Residual endotoxins [EU/mg]	0.044	0.000	0.003	0.055	0.012	**0.023**	±	**0.025**
Residual protein by Bradford assay [%]	< LoR[a]	< LoR	< LoR	< LoR	< LoR	**< 0.0017**		

AVE = average; LNT = lacto-*N*-tetraose; SD = standard deviation.
[a] LoR = 0.0017% (w/w).

2.3.3.3 Absence of Production Organism and its DNA

The production microorganism is efficiently removed by the ultrafiltration (Step 5) during USP, which is applied directly after fermentation. Various sequential filtration and purification processes are applied during DSP to ensure the final purity of LNT (Table 2.2.2-1). Absence of the production microorganisms in the bulk product is demonstrated by testing of final batches for bacteria from the *Enterobacteriaceae* family according to internationally-recognised methods (ISO 21528-1:2004, MSZ ISO 21528-2:2004). The ISO 21528-1:2004 method includes a pre-enrichment step to allow for resuscitation of the microorganism before enrichment.

The absence of the production organism in the finished ingredient is also supported by analyses for residual DNA in final production batches using 3 different validated qPCR methods (Table 2.3.3.3-1). Glycom's qPCR methods target short sub-sequences of the inserted genes as well as a short sub-sequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. Analysis of 5 independent batches of LNT product demonstrate no detectable levels of residual DNA (limit of quantification of 4 µg/kg or 4 ppb) in the final ingredient.

Table 2.3.3.3-1 Levels of Residual DNA in 5 Batches of LNT Produced by Fermentation

Parameters	Manufacturing Batch Numbers:							
	CPN4215 1000216 FD	CPN4215 1000316 FD	CPN4215 1000416 FD	CPN4215 1000516 FD	CPN4215 1000616 FD	AVE	±	SD
Residual DNA by qPCR (*lgtA* assay)	< LOQ[a]	< LOQ	< LOQ	< LOQ	< LOQ	**< LOQ**		
Residual DNA by qPCR (*galTK* assay)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	**< LOQ**		
Residual DNA by qPCR (23S assay)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	**< LOQ**		

DNA = deoxyribonucleic acid; LNT = lacto-*N*-tetraose; LOQ = limit of quantitation; qPCR = quantitative polymerase chain reaction.
[a] LOQ = 4 µg/kg (parts per billion).

2.3.3.4 Residual Anions, Trace Elements and Heavy Metals

Due to the nature of the fermentation process, the LNT preparation would theoretically have a potential to contain trace elements and minerals (as carry-over from the fermentation medium). However, the use of nanofiltration and ion-exchange is sufficient to reduce any appreciable carry-over of minerals from fermentation into the final ingredient. The results of trace element analyses are presented in Table 2.3.3.4-1. The averaged sum of sulphated ash does not exceed 0.10%; therefore, trace elements were not considered as compositional data of LNT. In addition, the trace element measurements included the confirmation that toxic heavy metals (such as lead) are not present.

Table 2.3.3.4-1 Levels of Anions, Trace Elements and Heavy Metals in 5 Batches of LNT Ingredient Produced by Fermentation

Parameter	Manufacturing Batch Numbers:							
	CPN4215 1000216 FD	CPN4215 1000316 FD	CPN4215 1000416 FD	CPN4215 1000516 FD	CPN4215 1000616 FD	AVE	±	SD
Orthophosphate by UV [%]	< 0.0009	0.0020	0.0020	0.0025	0.0022	**0.0019**	±	**0.0006**
Sulfate by IC [%]	0.1060	0.0077	0.0780	0.0520	0.0770	**0.0641**	±	**0.0369**
Sodium (Na) [mg/kg]	100	150	160	160	140	**142**	±	**25**
Potassium (K) [mg/kg]	20	30	30	30	20	**26**	±	**5**
Magnesium (Mg) [mg/kg]	30	10	30	< 10	10	**18**	±	**11**
Iron (Fe) [mg/kg]	1	5	1	2	2	**2**	±	**2**
Copper (Cu) [mg/kg]	< 0.1	< 0.1	< 0.1	< 0.2	< 0.1	**< 0.1**		
Manganese (Mn) [mg/kg]	0.2	< 0.1	< 0.1	< 0.1	0.1	**0.1**	±	**0.0**
Lead (Pb) [mg/kg]	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	**< 0.1**		
Ash, sulphated [%]	0.15	0.11	0.08	0.07	0.09	**0.10**	±	**0.03**

AVE = average; IC = ion chromatography; LNT = lacto-*N*-tetraose; SD = standard deviation; UV = ultraviolet.

2.4 Stability

Storage (real-time and accelerated) and stressed (forced) stability studies on the pure ("bulk") powdered LNT product were conducted by Wessling in accordance to the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Guidelines (*Stability Testing of New Drug Substances and Products*), (ICH, 2003) in order to:

i) test the LNT stability during storage;
ii) investigate degradation pathways when exposed to selected stress factors;
iii) define the optimal storage conditions and corresponding re-test dates or shelf-lives.

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

Stability studies in processed foods included new studies in powdered infant formula and previous studies with LNnT in powdered infant formula and other foods.

2.4.1 Bulk Stability

2.4.1.1 Real-Time Stability

The bulk stability of the LNT produced from fermentation, as described herein, was investigated under real-time conditions [25°C, 60% relative humidity (RH)] and accelerated conditions (40°C, 75% RH). A real-time 5-year stability study and a 2-year accelerated stability study are currently ongoing on representative batches of LNT. The chemical, physical, microbiological, and sensory testing was performed in an ongoing 5-year storage study (25°C, 60% RH) on 2 representative batches (No. CPN4215 00115 and CPN4215 1000516 FD), with interim results available up to 24 and 9 months, respectively, at the time of filing (see Table 2.4.1.1-1). The results further confirm that the ingredient is stable when stored at ambient room temperature for at least 24 months.

Table 2.4.1.1-1 Results of the 5-Year Real-Time Stability Study on LNT Product (25°C, 60% Relative Humidity, RH) for 2 Representative Batches: A) Batch No. CPN4215 00115, and B) Batch No. CPN4215 1000516 FDE

A) Manufacturing Batch Number CPN4215 00115

Parameter	Sample Time (Months)								
	0	1	2	3	6	9	12	18	24
Physical Properties									
Colour	White	White	White	White	White	White	Ivory white	Ivory white	Ivory white
Appearance	Not agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder	Agglutinated powder	Agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder
Purity									
Water content [%]	13.6	11.7	12.7	11.8	13.1	13.0	13.5	11.4	13.4
Assay – LNT [%]	78.3	77.0	77.0	78.0	78.0	78.6	78.3	77.7	76.3
Lactose [%]	1.74	1.89	1.83	1.96	1.90	1.94	2.07	1.88	1.90
Lactulose [%]	n.r.	< 0.1	< 0.1	< 0.1	< 0.1	0.10	< 0.1	< 0.1	< 0.1
Lacto-*N*-triose II [%]	4.00	4.52	4.42	4.46	4.13	4.58	4.82	3.91	3.50
Para-LNH2 [%]	0.68	0.72	0.61	0.68	0.65	0.65	0.67	0.73	0.70
LNT fructose isomer [%]	0.38	0.39	0.36	0.37	0.34	0.37	0.44	0.38	0.40
Assay (water free) HiMS [%]	84.9	84.3	84.2	85.3	84.9	86.1	86.2	84.3	82.5
Microbiological Quality									
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Salmonella spp.	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Listeria monocytogenes	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10

B) Manufacturing Batch Number CPN4215 1000516 FD

Parameter	Sample Time (Months)								
	0	1	2	3	6	9	12	18	24
Physical Properties									
Colour	White	White	White	White	White	White	Not tested	Not tested	Not tested
Appearance	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Not tested	Not tested	Not tested
Purity									
Water content [%]	2.4	2.3	2.3	2.2	1.8	2.0	Not tested	Not tested	Not tested
Assay – LNT [%]	74.9	75.0	75.7	77.7	76.9	74.4	Not tested	Not tested	Not tested
Lactose [%]	8.7	8.6	9.1	9.2	9.0	8.2	Not tested	Not tested	Not tested
Lactulose [%]	0.2	0.2	0.2	0.2	0.2	0.2	Not tested	Not tested	Not tested
Lacto-*N*-triose II [%]	5.7	5.9	5.8	5.6	5.6	5.6	Not tested	Not tested	Not tested
Para-LNH2 [%]	n.a.	2.0	2.0	2.0	2.2	2.0	Not tested	Not tested	Not tested
LNT fructose isomer [%]	1.0	1.0	0.8	1.0	0.9	1.0	Not tested	Not tested	Not tested
Assay (water free) HiMS [%]	89.7	89.8	91.0	92.8	91.8	88.5	NA	NA	NA
Microbiological Quality									
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Salmonella spp.	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Listeria monocytogenes	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested

CFU = colony-forming units, LNT = lacto-*N*-tetraose, GlcNAc = N-acetyl-D-glucosamine, Gal = galactose, HiMS = human-identical milk saccharides = sum of LNT, lactose and lacto-*N*-triose II; NA = not applicable; n.a. = not available; n.r. = not reported; para-LNH2 = para-lacto-*N*-hexaose-2.

2.4.1.2 Accelerated Stability

The bulk stability of spray dried, amorphous LNT products was investigated, under accelerated conditions (40°C, 75% RH) for a period of 2 years. The results for 2 representative batches No CPN4215 00115 and No CPN4215 1000516 FD are presented in Table 2.4.1.2-1 below. The results of these studies indicate that there are no changes in organoleptic properties of LNT, no appreciable degradation of LNT, no changes in impurity profile, and no alterations in the microbiological quality of the ingredient following storage for up to 2 years under defined, accelerated storage conditions. LNT was analysed by HPLC and water content was analysed by Karl Fischer titration at each time point.

Two independent lots of LNT were demonstrated to be stable throughout the 24- and 9-month storage periods respectively (for Batch Nos. CPN4215 1000516 FD and CPN4215 1000516 FD, respectively) with no measurable loss of LNT, other carbohydrates or change in impurities content. As with the real-time stability testing, no appreciable changes, degradation of the ingredient, or alterations in impurity profiles were reported. Based on the results of the accelerated stability study and using the Arrhenius equation (Peleg *et*

al., 2012), the stability of the ingredient was calculated to be at least 5 years when protected from light and stored at room temperature and ambient humidity.

Table 2.4.1.2-1 Representative Interim Results of the 2-Year Accelerated Stability Study on LNT Product (40°C, 75% Relative Humidity, RH) for 2 Representative Batches: A) Batch No. CPN4215 00115, and B) Batch No. CPN4215 1000516 FD

A) Manufacturing Batch Number CPN4215 00115

Parameter	Sample Time (Months)								
	0	1	2	3	6	9	12	18	24
Physical Properties									
Colour	White	White	White	White	White	White	Ivory white	Ivory white	Ivory white
Appearance	Not agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder	Agglutinated powder	Agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder
Purity									
Water content [%]	13.6	11.7	12.7	11.8	13.1	13.0	13.5	11.4	13.4
Assay – LNT [%]	78.3	77.0	77.0	78.0	78.0	78.6	78.3	77.7	76.3
Lactose [%]	1.74	1.89	1.83	1.96	1.90	1.94	2.07	1.88	1.90
Lactulose [%]	n.r.	< 0.1	< 0.1	< 0.1	< 0.1	0.10	< 0.1	< 0.1	< 0.1
Lacto-*N*-triose II [%]	4.00	4.52	4.42	4.46	4.13	4.58	4.82	3.91	3.50
Para-LNH2 [%]	0.68	0.72	0.61	0.68	0.65	0.65	0.67	0.73	0.70
LNT fructose isomer [%]	0.38	0.39	0.36	0.37	0.34	0.37	0.44	0.38	0.40
Assay (water free) HiMS [%]	84.9	84.3	84.2	85.3	84.9	86.1	86.2	84.3	82.5
Microbiological Quality									
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Salmonello spp.	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Listeria monocytogenes	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10

B) Manufacturing Batch Number CPN4215 1000516 FD

Parameter	Sample Time (Months)								
	0	1	2	3	6	9	12	18	24
Physical Properties									
Colour	White	White	White	White	White	White	Not tested	Not tested	Not tested
Appearance	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Not tested	Not tested	Not tested
Purity									
Water content [%]	2.4	2.3	2.3	2.2	1.8	2.0	Not tested	Not tested	Not tested
Assay – LNT [%]	74.9	75.0	75.7	77.7	76.9	74.4	Not tested	Not tested	Not tested
Lactose [%]	8.7	8.6	9.1	9.2	9.0	8.2	Not tested	Not tested	Not tested
Lactulose [%]	0.2	0.2	0.2	0.2	0.2	0.2	Not tested	Not tested	Not tested
Lacto-*N*-triose II [%]	5.7	5.9	5.8	5.6	5.6	5.6	Not tested	Not tested	Not tested
Para-LNH2 [%]	n.a.	2.0	2.0	2.0	2.2	2.0	Not tested	Not tested	Not tested
LNT fructose isomer [%]	1.0	1.0	0.8	1.0	0.9	1.0	Not tested	Not tested	Not tested
Assay (water free) HiMS [%]	89.7	89.8	91.0	92.8	91.8	88.5	NA	NA	NA
Microbiological Quality									
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Salmonella spp.	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Listeria monocytogenes	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested

CFU = colony-forming units, LNT = lacto-*N*-tetraose, GlcNAc = N-acetyl-D-glucosamine, Gal = galactose; HiMS = human-identical milk saccharides = sum of LNT, lactose and lacto-*N*-triose; NA = not applicable; n.a. = not available; n.r. = not reported; para-LNH2 = para-lacto-*N*-hexaose-2.

2.4.1.3 Stress/Forced Stability

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

Forced stability tests of the bulk LNT product in aqueous solutions were performed at 60 and 80°C for 8 and 4 weeks of storage, respectively, using dissolved:

- Amorphous LNT powder at slightly acidic pH (4.5)
- Crystalline LNT powder at neutral pH (6.3) as reference
- Amorphous LNT powder at neutral pH (6.8)

The results of this study showed the presence of 2 potential pH-dependent chemical degradation pathways in the aqueous solutions of LNT product, namely hydrolysis at pH < 5.0 and isomerisation at pH > 6.0. At

neutral pH LNT undergoes minor isomerisation to the LNT fructose isomer for both LNT products (in crystalline and amorphous form). At slightly acidic pH, amorphous LNT was hydrolysed to glucose and lacto-*N*-triose II. It has been concluded that the optimal stability of LNT powder is observed at a pH between 5 and 6 (optimum close to 5.5), where neither the hydrolysis processes (occurring below pH 5) nor the isomerisation process (occurring above pH 6) is pronounced.

2.4.2 Stability Under the Intended Conditions of Use

2.4.2.1 Stability in Powdered Infant Formula

The stability of LNT in powdered infant formula was investigated following long-term storage at temperatures of 4, 20, 30 and 37°C.

The infant formula powder tested was a whey-based commercially available starter formula supplemented with LNT, which was formulated together with other HiMOs to simulate their intended conditions of use in infant formula. The infant formula also contained long chain polyunsaturated fatty acids (LC-PUFA), and vitamins and minerals at concentrations intended for full nutritional support of infants from birth to 6 months of age. The interim results available to 12 months are presented in Table 2.4.2.1-1. The results suggest alignment between expected and observed values of the selected HMO (LNT), and, as a result, stability of these compounds after 12 months storage under the test conditions.

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

			LNT
	Target	g/100g IF	0.74
Sample time (months) and temperature (°C)	T0		0.85
	3 months	4°C	0.85
		20°C	0.85
		30°C	0.84
		37°C	0.84
	6 months	4°C	0.84
		20°C	0.87
		30°C	0.85
		37°C	0.85
	9 months	4°C	0.94
		20°C	0.92
		30°C	0.90
		37°C	0.90
	12 months	4°C	0.88
		20°C	0.88
		30°C	0.89
		37°C	0.88

IF = infant formula; LNT = lacto-*N*-tetraose.
* Targeted concentration of HiMO per 100 g of IF.

2.4.2.2 *Stability in Other Food Matrices*

LNT and LNnT are constitutional isomers (or "linkage" isomers), where the Gal of the non-reducing terminus is bonded to GlcNAc *via* a β1-3 linkage in LNT (type 1), and a β1-4 linkage (type 2) in LNnT (Yamagaki *et al.*, 2006). Based on the highly similar structure, comparable properties for LNnT and LNT in terms of stability in food can be assumed.

It has been demonstrated that LNnT is stable in various food matrices, including yoghurts, ready-to-drink flavoured milk, and citrus fruit beverages, following typical processing conditions and when stored at 4°C over the shelf-life of these foods (this data was evaluated during the LNnT novel food application, also see GRAS Notice (GRN) 547 for analytical results; U.S. FDA, 2015). All stability studies were conducted using formulations representative of commercial food products on the market and under typical processing (*i.e.*, pasteurisation and/or UHT heating) and typical storage conditions (*e.g.*, temperature and shelf-life) for such products.

Part 3. § 170.235 Dietary Exposure

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

3.1.1 LNT

To the best of our knowledge, the LNT product described herein has not been previously concluded to have GRAS status for food use in the U.S.

3.1.2 Other Manufactured HiMOs

The structurally related isomer, LNnT, produced by chemical synthesis or by fermentation has been concluded to have GRAS status in the U.S., and is authorized as a novel food in the EU, Israel, and Singapore.

3.1.2.1 *United States*

LNnT Produced by Fermentation

Glycom's LNnT ingredient obtained by fermentation using an *Escherichia coli* K 12-derived strain has GRAS status for use in term infant formula at a maximum level of 0.6 g LNnT per litre, as well as for use in various other foods [including other baby foods and drinks for infants and young children such as toddler formula, beverages and beverage bases, dairy product analogues, grain products and pastas, milk (whole and skim), milk products, processed fruits and juices, and processed vegetables and juices] at maximum levels ranging from 0.02 to 0.68 g of LNnT per serving. The GRAS status of LNnT for use in specified conventional foods and infant formula was notified to the offices of the U.S. Food and Drug Administration (FDA) in 2016 (GRN 659) and the filed by the FDA without objection under the GRN659 (U.S. FDA, 2016b).

3.1.2.2 *EU*

LNnT manufactured by Glycom from both synthetic and microbial processes has been approved for use in a number of food applications under Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods. The abovementioned Union List of Novel Foods includes (but is not limited to):

i) The previous EU formal approval obtained by Glycom A/S under Regulation (EC) No 258/97, Commission Implementing Decisions (EU) 2016/375 and 2016/376 of 11 March 2016 authorising the placing on the market of lacto-*N*-neotetraose as a novel food ingredient. This authorization was based on the EFSA Scientific opinion on the safety of lacto-*N*-neotetraose as novel food ingredient, and the EFSA Statement on the safety of lacto-*N*-neotetraose

ii) as novel food ingredients in food supplements for children (EFSA NDA Panel, 2015a,b).

iii) The successful Substantial Equivalence Notifications of Glycom for the same uses for lacto-*N*-neotetraose from the microbial source "Genetically modified strain of *Escherichia coli* K-12", which was based on the Scientific Opinion from Food Safety Authority of Ireland (FSAI, 2016).

3.1.3 History of Consumption in Breast Milk

3.1.3.1 Human Biology Background Relevant to LNT

LNT is an important component of the natural HMO fraction of human milk. Human milk contains, as its third largest solid component, a fraction consisting of a complex family of structurally-related oligosaccharides (Kuhn, 1952; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013). These are known as HMOs because they were first discovered in human breast milk (Malpress and Hytten, 1958) and are present at much higher concentrations than in any other mammalian milk (Urashima *et al.*, 2001). More than 140 members of this family have been fully described on a structural basis (Chen, 2015; Urashima *et al.*, 2018), and an even higher number of members have been detected by sensitive mass spectrometry techniques (Finke *et al.*, 1999; Wu *et al.*, 2010, 2011). The highest concentrations of HMOs occur in human colostrum (20 to 25 g/L), and concentrations between 5 to 20 g/L occur in mature human milk (Bode, 2012) although high variations are reported on individual level and in dependency of the lactation period and the genotype of the mother. Bovine colostrum contains approximately 20 times less of a far less complex oligosaccharide mixture and mature cow milk contains only traces (Tao *et al.*, 2009; Aldredge *et al.*, 2013; Urashima *et al.*, 2013). The respective composition of each mammalian milk oligosaccharide fraction allows interesting insights into evolutionary aspects of lactation (Urashima *et al.*, 2012).

LNT belongs to the "neutral core" sub-fraction of HMOs, oligosaccharides that contain the aminosugar *N*-acetyl-D-glucosamine (GlcNAc) and which is reported to constitute on average around 15% of the total HMO fraction (Bode *et al.*, 2012). The "core" HMOs can be further decorated by fucose or sialic acid, forming fucosylated and sialylated HMOs fractions, respectively. It has been established that the HMO pattern depends on the genetic background of the mother, with some variations observed in regard to the fucosylation patterns. However, "core" HMOs, like LNT and LNnT, are present in all types of milks from all mother's phenotypes (Kunz *et al.*, 2017). The biological relevance of the structural difference between LNT and LNnT, *i.e.*, type 1 vs type 2 linkage between the terminal Gal and next to last GlcNAc (and the respective ratio between the 2 forms) is not fully understood; however, it is equally observed in important cell-surface carbohydrate antigens like the blood group and Lewis type antigens, and glycosphingolipids & gangliosides[11] (Yu *et al.*, 2007; Hod *et al.*, 2009). It is noteworthy to mention that LNT and LNnT are metabolized by a different set of human gut commensal microbes that possess highly specific enzymes that allow them to utilize either one or the other HMO (Katayama, 2016; Özcan and Sela, 2018), a process that has been discussed to be relevant to the evolution of human-microbe symbiosis (Yamada *et al.*, 2017). Indeed, it had

[11] The linkage difference between LNT and LNnT (*i.e.*, Gal-ß(1-**3**)-GlcNAc vs Gal-ß(1-**4**)-GlcNAc) is for historical reasons called "type 1" and "type 2" in blood group and Lewis antigen research, while the identical structural difference is called "lacto" and "neolacto" series in glycolipid research.

been previously observed that milk oligosaccharides of type 1 (*e.g.*, LNT) predominate over type 2 (*e.g.*, LNnT) in human milk, while the inverse is the case in all other mammalian milks (Urashima *et al.*, 2012)

3.1.3.2 Quantity of LNT in Breast Milk

The concentration of LNT in human milk has been measured and reported to date in at least 18 independent publications (Table 3.1.2.2-1). The data demonstrate that LNT is, on average, the most abundant "neutral-core" HMO of pooled human milk and among the most abundant HMOs in general.

The average LNT levels in pooled milk remain relatively stable throughout the lactation period, achieving concentrations of 0.8 g/L in colostrum, followed by 0.9 g/L and 1.1 g/L in transitional and mature milks, respectively; concentrations measured in mature milk gradually decline in samples obtained from a lactation stage later than 2 months (0.5 g/L). The reported ranges differ widely between 0.1 and 3.7 g/L. Some studies report even significantly higher levels, but have been considered as outliers for this analysis, as these studies were all connected to the same group of investigators and are inconsistent with numerous reports by a several independent researchers that have applied a set of different analytical methods (Gabrielli *et al.*, 2011; Galeotti *et al.*, 2012, 2014; Bao *et al.*, 2013).

Table 3.1.2.2-1 Reported LNT Concentration in Human Milk after Full-Term Birth

Lactation Time	Key Findings	References
Pooled Milk		
Days 1 to 4 ("colostrum")	**Reported Range: 0.3 to 1.7 g/L** Average: 0.8 ± 0.4 g/L	Coppa *et al.* (1999); Erney *et al.* (2000); Kunz *et al.* (2000, 2017); Sumiyoshi *et al.* (2003); Asakuma *et al.* (2008); Thurl *et al.* (2010); Spevacek *et al.* (2015); Aakko *et al.* (2017)
Days 5 to 14 ("transitional milk")	**Reported Range: 0.4 to 1.5 g/L** Average: 0.9 ± 0.4 g/L	Coppa *et al.* (1999); Erney *et al.* (2000); Sumiyoshi *et al.* (2003); Kunz *et al.* (2000); Spevacek *et al.* (2015); Austin *et al.* (2016)
Days 10 to 60 ("mature milk")	**Reported Range: 0.1 to 2.3 g/L** Average: 1.1 ± 0.4 g/L	Chaturvedi *et al.* (1997); Coppa *et al.* (1999); Erney *et al.* (2000, 2001); Sumiyoshi *et al.* (2003); Thurl *et al.* (2010); Asakuma *et al.* (2011); Hong *et al.* (2014); Olivares *et al.* (2015); Spevacek *et al.* (2015); Austin *et al.* (2016); McGuire *et al.* (2017); Sprenger *et al.* (2017)
After 2 months ("mature milk")	**Reported Range: 0.3 to 1.3 g/L** Average: 0.5 ± 0.4 g/L	Coppa *et al.* (1999); Smilowitz *et al.* (2013); Austin *et al.* (2016); Sprenger *et al.* (2017)

LNT = lacto-*N*-tetraose.

A recently published systematic review of the concentrations of oligosaccharides in human milk from secretor mothers (Thurl *et al.*, 2017) reported the content of LNT at 0.79 g/L (with 95% confidence limits of 0.59 to 0.98). In milk from secretor mothers, who delivered full-term, the corresponding LNT levels (1.0 ± 0.2 g/L) are on average lower than in the milk of non-secretor mothers (1.7 ± 0.7 g/L), based on 6 further independent studies (Thurl *et al.*, 2010; Hong *et al.*, 2014; Kunz *et al.*, 2017; McGuire *et al.*, 2017; Sprenger *et al.*, 2017) and suggesting a possible co-regulation of secretor function and LNT expression. A possible explanation for this difference is that in the milk of secretor mothers with its additional FUT2 fucosyltransferase, LNT is a preferential substrate to yield a diversity of additional mono- and difucosylated HMOs, adding to the complexity of the oligosaccharide fraction, but concomitantly reducing the LNT levels (Kunz *et al.*, 2003).

Data from different regions of the world is available (and summarized in Figure 3.1.2.2-1), but only 2 studies have investigated the regional (ethnic) dependency of the LNT concentration of milk under comparable conditions (Erney *et al.*, 2000; McGuire *et al.*, 2017). Under comparable conditions insignificant differences of average LNT concentrations were observed between Asia, Europe, Latin America, and the U.S. (Erney *et*

al., 2000). The African population samples appear to possess increased levels of LNT in their milk (1.2 g/L); however, this data is based on a single study (McGuire *et al.*, 2017). Therefore, it is possible that the reported low and high extremes may be study-biased (with major confounders like time of sampling and analytical methods), rather than real differences. The most parsimonious conclusion is that there is a wide variation between individual mothers that covers ranges up to more than 1 g/L of LNT.

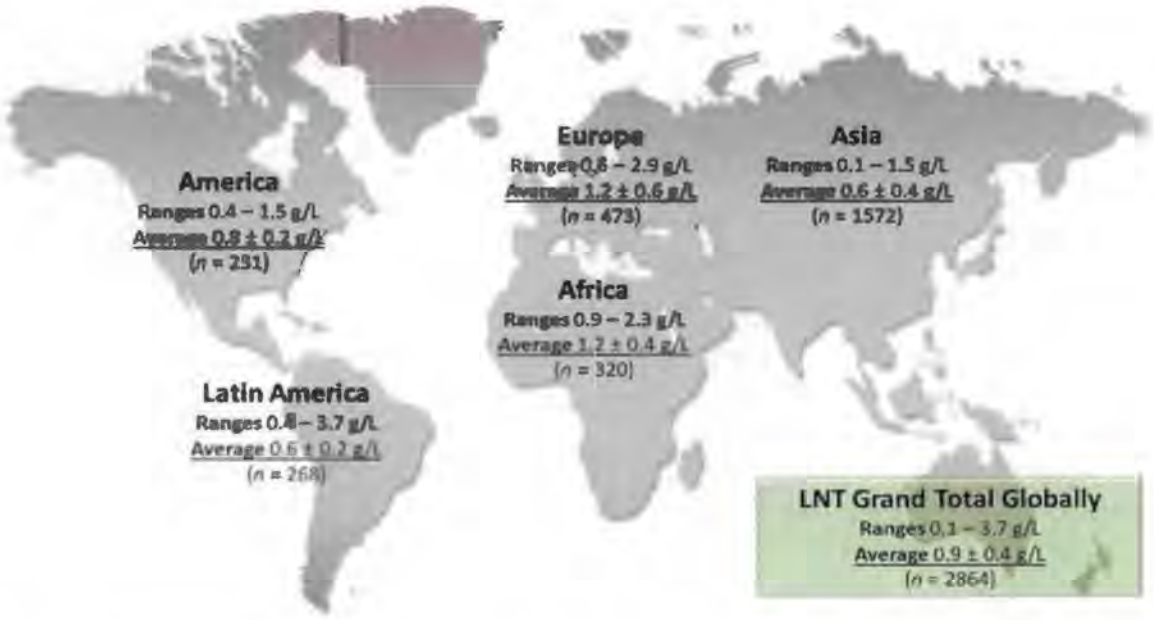


Figure 3.1.2.2-1 Reported LNT Ranges and Average LNT Concentrations (g/L) in Pooled, Mature Human Milk from Mothers Delivered Term by Geographic Regions
(based on data from: Chaturvedi *et al.*, 1997, 2001; Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Thurl *et al.*, 2010; Asakuma *et al.*, 2011; Smilowitz *et al.*, 2013; Hong *et al.*, 2014; Olivares *et al.*, 2015; Spevacek *at al.*, 2015; Austin *et al.*, 2016; Kunz *et al.*, 2017; Sprenger *et al.*, 2017; McGuire *et al.*, 2017).

Using the average levels of LNT reported for breast milk, of 0.5 to 1.1 g/L (see Table 3.1.2.2-1), combined with the estimated formula intake value for young infants of 260 mL/kg body weight/day (EFSA, 2017), the intake level of LNT from the diet can be estimated at between 130 to 286 mg/kg body weight/day.

Based on the reported ranges, with up to 3.7 g/L measured in mature milk, LNT intakes may be measured at up to 962 mg/kg body weight/day, using the same assumptions as above for infant formula intake (260 mL/kg body weight/day).

3.1.4 History of Commercial Use

3.1.4.1 LNT

To best of our knowledge, LNT has not been marketed as a food ingredient.

3.1.4.2 Related HiMOs

Other HiMOs produced by fermentation from a modified *E. coli* strain, have been commercialized as ingredients of milk-based infant formulas worldwide. As example, 2 infant formulas under the name NAN

Optipro Supreme containing 2'-FL and LNnT (lacto-*N*-neotetraose) were launched by Nestle in Spain and Portugal in 2017 and as BEBA Supreme in Germany in 2018.

Europe and Asia – Spain, Portugal, Germany and Hong Kong – Nestle (Glycom)

NAN Optipro Supreme 1 HM-0[2], designed for children from 0 months old, containing 2 oligosaccharides designed with a structure identical to those found in human milk. 2'-fucosyl-lactose (2'-FL): 0.1 g/100 mL and lacto-*N*-neotetraose (LNnT): 0.05 g/100 mL.

NAN Optipro Supreme 2 HM-0[2], containing 2 oligosaccharides designed with a structure identical to those found in human milk 2'-fucosyl-lactose (2'-FL): 0.1 g/100 mL and Lacto-*N*-neotetraose (LNnT): 0.05 g/100 mL.

3.2 Estimated Intake of LNT

3.2.1 Methods

An assessment of the anticipated intake of LNT as an ingredient under the intended conditions of use (see Table 1.3-1) was conducted using data available in the 2013-2014 cycle of the U.S. National Center for Health Statistics (NCHS)'s National Health and Nutrition Examination Survey (NHANES) (CDC, 2015, 2016; USDA, 2016). A summary along with the pertinent results is presented herein.

The NHANES data are collected and released in 2-year cycles with the most recent cycle containing data collected in 2013 to 2014. Information on food consumption was collected from individuals *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2). Sample weights were incorporated with NHANES data to compensate for the potential under-representation of intakes from specific populations and allow the data to be considered nationally representative (CDC, 2016; USDA, 2016). The NHANES data were employed to assess the mean and 90th percentile intake of LNT for each of the following population groups:

- Toddlers, ages 1 to 3;
- Children, ages 4 to 10;
- Female teenagers, ages 11 to 18;
- Male teenagers, ages 11 to 18;
- Female adults of childbearing age, ages 19 to 40;
- Female adults, ages 19 to 64;
- Male adults, ages 19 to 64;
- Elderly, ages ≥ 65; and
- Total population (all age and gender groups combined).

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of LNT by the U.S. population[12]. Estimates for the daily intake of LNT represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2013-2014; these average amounts comprised the distribution from which mean, and percentile intake estimates were determined. Mean and percentile estimates were generated incorporating survey weights to provide representative intakes for the entire U.S. population. *"Per capita"* intake refers to the estimated intake of LNT averaged over all individuals surveyed, regardless of whether they consumed food products in which LNT is proposed for use, and therefore includes individuals with "zero" intakes (*i.e.*, those who reported no intake of food products containing LNT during the 2 survey days). "Consumer-only" intake refers to the estimated intake of LNT by those individuals who reported consuming food products in which the use of LNT is currently under consideration. Individuals were considered "consumers" if they reported consumption of 1 or more food products in which LNT is proposed for use on either Day 1 or Day 2 of the survey.

The estimates for the intake of LNT were generated using the maximum use-level indicated for each intended food-use, as presented in Table 1.3-1, together with food consumption data available from the 2013-2014 NHANES datasets. The results of this assessment is presented in Section 3.2.2.

3.2.2 Intake Estimates for LNT

A summary of the estimated daily intake of LNT from proposed food-uses is provided in Table 3.2.2-1 on an absolute basis (g/person/day), and in Table 3.2.2-2 on a body weight basis (mg/kg body weight/day). Intakes are expressed as total wet weight of the ingredient under the conditions of intended use.

The percentage of consumers was high among all age groups evaluated in the current intake assessment; more than 92.2% of the population groups consisted of consumers of food products in which LNT is currently proposed for use (Table 3.2.2-1). Children had the greatest proportion of consumers at 99.0%. The consumer-only estimates are more relevant to risk assessments as they represent exposures in the target population; consequently, only the consumer-only intake results are discussed in detail herein.

Among the total population (all ages), the mean and 90th percentile consumer-only intakes of LNT were determined to be 0.83 and 1.77 g/person/day, respectively. Of the individual population groups, male adults were determined to have the greatest mean and 90th percentile consumer-only intakes of LNT on an absolute basis, at 0.94 and 2.02 g/person/day, respectively. The elderly had the lowest mean consumer-only intake of 0.66 g/person/day, while female teenagers had the lowest 90th percentile consumer-only intakes of 1.37 g/person/day (Table 3.2.2-1).

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

Population Group	Age Group (Years)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Toddlers	1 to 3	0.89	1.86	98.5	465	0.90	1.88
Children	4 to 10	0.73	1.46	99.0	986	0.74	1.47
Female Teenagers	11 to 18	0.66	1.37	94.5	572	0.70	1.37

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

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

Population Group	Age Group (Years)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Male Teenagers	11 to 18	0.91	1.62	98.2	570	0.93	1.66
Female Adults of Childbearing Age	19 to 40	0.72	1.56	92.9	826	0.78	1.57
Female Adults	19 to 64	0.73	1.69	92.9	1,764	0.79	1.72
Male Adults	19 to 64	0.87	1.97	92.7	1,522	0.94	2.02
Elderly	65 and up	0.61	1.49	92.2	917	0.66	1.53
Total Population	All ages	0.78	1.72	93.8	7,088	0.83	1.77

LNT = lacto-*N*-tetraose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.
*Intake data expressed as wet weight of ingredient under the proposed conditions of intended use.

On a body weight basis, the total population (all ages) mean and 90th percentile consumer-only intakes of LNT were determined to be 17.2 and 33.6 mg/kg body weight/day, respectively. Among the individual population groups, toddlers were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, of 70.2 and 141.0 mg/kg body weight/day, respectively. The elderly had the lowest mean and 90th percentile consumer-only intakes of 8.8 and 20.6 mg/kg body weight/day, respectively (Table 3.2.2-2).

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

Population Group	Age Group (Years)	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Toddlers	1 to 3	69.1	141.0	98.5	460	70.2	141.0
Children	4 to 10	27.4	56.0	98.9	980	27.7	56.4
Female Teenagers	11 to 18	11.6	26.0	94.6	568	12.3	26.4
Male Teenagers	11 to 18	14.3	27.9	98.2	569	14.6	27.9
Female Adults of Childbearing Age	19 to 40	10.0	22.1	92.9	819	10.7	22.8
Female Adults	19 to 64	9.8	22.7	92.9	1,752	10.6	23.2
Male Adults	19 to 64	10.2	23.8	92.7	1,518	11.0	24.9
Elderly	65 and up	8.1	20.4	92.1	906	8.8	20.6
Total Population	All ages	16.2	32.0	93.8	7,045	17.2	33.6

LNT = lacto-*N*-tetraose; bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.
*Intake data expressed as wet weight of ingredient under the proposed conditions of intended use.

3.2.3 Summary and Conclusions

Consumption data and information pertaining to the individual proposed food-uses of LNT were used to estimate the *per capita* and consumer-only intakes of LNT for specific demographic groups and for the total U.S. population. There were a number of assumptions included in the assessment which render exposure estimates that may be considered suitably conservative. For example, it has been assumed in both exposure assessments that all food products within a food category contain LNT at the maximum specified level of use. In reality, the levels added to specific foods will vary depending on the nature of the food product and it is unlikely that LNT will have 100% market penetration in all identified food categories.

In summary, on consumer-only basis, the resulting mean and 90th percentile intakes of LNT by the total U.S. population from all proposed food-uses, were estimated to be 0.83 g/person/day (17.2 mg/kg body weight/day) and 1.77 g/person/day (33.6 mg/kg body weight/day), respectively. Among the individual population groups, the highest mean and 90th percentile consumer-only intakes of LNT were determined to be 0.94 g/person/day (11.0 mg/kg body weight/day) and 2.02 g/person/day (24.9 mg/kg body weight/day), respectively, as identified among male adults. The elderly had the lowest mean consumer-only intakes of 0.66 g/person/day (8.8 mg/kg body weight/day), while female teenagers had the lowest 90th percentile consumer-only intakes of 1.37 g/person/day (26.4 mg/kg body weight/day). When intakes were expressed on a body weight basis, toddlers had the highest mean and 90th percentile consumer-only intake of 70.2 and 141.0 mg/kg body weight/day.

Glycom notes that GRAS status through scientific procedures should include consideration of the probable consumption of the substance and the probable consumption of any "*... chemically or pharmacologically related substances in the diet.*" (81 FR 54959 – U.S. FDA, 2016c). In this regard Glycom notes that LNT may be used with other HiMOs, including, but not limiting to, 2'-FL and LNnT, which already have GRAS status for use in non-exempt infant formula and select conventional food products. Glycom notes that potential for additive exposure to compounds with similar structure function activities in the diet of infants may occur; however, as LNT is intended to be added to infant formula at levels that are representative of human milk, the additive presence of other HiMOs at levels that are also naturally present in human milk will not be of safety concern.

Part 4. § 170.240 Self-Limiting Levels of Use

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

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 subject matter of this GRAS evaluation is lacto-*N*-tetraose (LNT). LNT manufactured by Glycom is identical in structure to its natural counterpart secreted into human milk and therefore can be referred to as a HiMO. The ingredient will be added to infant formula at levels that are consistent with levels of LNT that have been measured in breast milk samples across all lactational stages and therefore the safety of adding LNT to infant formula is principally based on the safe history of consumption of LNT by breast-feeding infants. As infants are a sensitive population group, the safety of dietary ingestion of HiMOs from breast milk consumption also can be extended to adults consuming HiMOs at comparable ingestion levels in conventional food products.

Studies characterizing the toxicity of LNT include a battery of published toxicity studies, including an acute 14-day toxicity study in neonatal Sprague-Dawley rats, a subchronic repeat-dose 90-day feeding study in neonatal Sprague-Dawley rats, a bacterial reverse mutation test, and an *in vitro* mammalian cell micronucleus test have been conducted using LNT preparations manufactured by Glycom. Findings from

these studies demonstrated that the ingredient is without evidence of toxicity in neonatal rats up to the highest dose tested and is not genotoxic or mutagenic. These studies are summarized in Section 6.4.1.

As mentioned previously, LNnT is a chemically and structurally similar isomer of LNT. Therefore, the toxicological data obtained for LNnT preparations are considered relevant in supporting the safety of LNT. Studies evaluating the toxicity of LNnT test articles manufactured by Glycom using chemical synthesis or microbial fermentation have been reviewed by various qualified Experts during previous GRAS evaluations as well as by the U.S. FDA (GRN 547 and GRN 659) and EFSA (for LNnT from chemical synthesis). The results of animal toxicity studies have consistently demonstrated that LNnT is without evidence of toxicity up to the highest dose tested, findings that are consistent with the safe history of consumption of LNnT by breastfed infants. Results of multiple genotoxicity batteries, including the Ames reverse mutation assay, *in vitro* chromosomal aberration assays, mouse lymphoma cell gene mutation test, and *in vitro/in vivo* micronucleus assays were all negative for evidence of genotoxicity/mutagenicity. These studies are summarized in Section 6.4.2.

Safety and tolerance studies in infants have not been conducted using LNT; however, there is no scientific basis to expect that the addition of LNT to infant formula at concentrations that are representative of levels in human milk would not similarly be safe and well tolerated. Findings from published clinical studies in infants and healthy adult subjects assessing the safety and tolerance of the structural isomer LNnT have been the subject of previous evaluations by various qualified experts during GRAS panel evaluations and novel food evaluations by EFSA (Section 6.5.2). Formula supplemented with 0.5 to 0.6 g LNnT (in combination with 1.0 to 1.2 g 2'-FL) for the first 4 months of life was well tolerated and supported age-appropriate growth (Puccio *et al.*, 2017). In adults, the results of a safety and tolerability study indicate that consumption of LNnT at doses of up to 20 g, 2'-FL at doses of up to 20 g, or their combination at a 2:1 ratio (for total sum of up to 20 g), when taken as a single bolus dose daily for 2 weeks, was well tolerated and did not result in any deviations in laboratory measures of safety (*i.e.*, hematology and blood biochemistry) compared to normal reference values (Elison *et al.*, 2016). Together, these clinical studies support the safety of LNnT, and indirectly the safety of LNT.

Finally, Glycom evaluated the allergenicity risk of LNT (Section 6.6). The amino acid sequences of all introduced proteins were evaluated using Allergen Online tool (version 18B) hosted by the University of Nebraska's Food Allergen Research and Resource Program (FARRP). No positive alignments between any of the recombinant proteins and known/putative allergen sequences within the database were identified.

6.2 Literature Search

Glycom considered the totality of publicly available data and information relevant to the safety of LNT. As LNT is a structural isomer of LNnT, comprehensive searches of the published scientific literature were therefore conducted from the last GRAS evaluation of LNnT in 2016 through October 2018. The search was conducted using the electronic search tool, ProQuest Dialog™, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and Toxfile®. Consistent with the requirements of the GRAS standard, conclusions on the GRAS status of LNT have considered all publicly available sources of information including favorable and potentially unfavorable information. Based on Glycom's updated search of the literature, the company is not aware of published studies to suggest the LNT is unsafe for use as a food ingredient.

6.3 Absorption, Distribution, Metabolism, and Excretion

LNT manufactured by Glycom is structure-identical to its naturally occurring counterpart in human milk. HMOs, including LNT, do not undergo any significant digestion in the upper gastrointestinal tract; however, HMOs are orally absorbed intact to a small extent, a small portion of which (approximately 1 to 2% of the total amount of HMO ingested) is excreted unchanged in urine. The absorption of the LNT would be limited and quantities of LNT that are absorbed would be no different to that occurring in infants consuming human breast milk. For a more comprehensive discussion of the ADME profile of LNT and other HiMOs, the reader is directed to Sections IV.B.4 of GRN 546 and IV.D of GRN 650 (U.S. FDA, 2015, 2016a).

6.4 Toxicology Studies

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

6.4.1 Studies Conducted with LNT

6.4.1.1 Repeat Dose Toxicity Studies

6.4.1.1.1 14-Day Toxicity Study in the Neonatal Rat

A 14-day repeat dose toxicity study was conducted in rats to evaluate the potential short-term toxicity of LNT and select dose levels for the subsequent 90-day study (Phipps *et al.*, 2018).

Groups of 8 male and 8 female neonatal rats were dosed with 0 (water for irrigation), 3,250 or 4,000 mg/kg body weight/day LNT, by gavage at a dose volume 10 mL/kg body weight, once daily for 14 days, until the day before necropsy. The high dose of 4,000 mg/kg body weight/day was the maximum feasible dose, based on viscosity of the test item formulation. Doses of LNT were corrected to account for "other carbohydrates" within the test article batch.

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

There were no test item-related deaths. One male receiving 4,000 mg/kg body weight/day was found dead on Day 14 of dosing. This animal had shown no changes in clinical condition; however, it gained slightly less weight (2%) than the other males in this group (8 to 11%) between Days 13 and 14 of dosing. Macroscopic examination revealed no abnormalities and there was no evidence of dosing trauma. As this was an

isolated incident, this premature death was considered incidental and unrelated to treatment with LNT. There were no test item-related clinical signs, no biologically relevant differences in body weight between test item-treated groups and controls and no test item-related macroscopic abnormalities at necropsy.

In absence of any test item-related adverse findings, 4,000 mg/kg body weight/day (the maximum feasible dose, based on viscosity) was considered the no-observed-adverse-effect level (NOAEL) and a suitable high-dose for the 90-day study.

6.4.1.1.2 90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of LNT when administered by gavage to neonatal rats from Day 7 of age (Phipps *et al.*, 2018). The study was conducted in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b), and included an adapted study design to incorporate the use of neonatal animals to consider the requirements of EFSA *Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age* (EFSA, 2017), Guidance for industry: nonclinical safety evaluation of paediatric drug products (U.S. FDA, 2006), Guideline on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications (EMEA, 2008) and the Guideline on the Nonclinical Safety Study in Juvenile Animals for Paediatric Drugs (MHLW, 2018).

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 1,000, 2,500, or 4,000 mg/kg body weight/day LNT, by gavage at a dose volume of 10 mL/kg body weight, once daily for at least 90 days, until the day before necropsy. An additional reference control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 4,000 mg/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose LNT group and identify any effects related to the general fibre-like characteristics of the reference material. A further 5 males and 5 females in each group were also dosed once daily for at least 90 days and then kept un-dosed for 4 weeks, to assess the reversibility of any observed effects.

Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of treatment until weaning and twice weekly thereafter. Food intake was recorded twice weekly from weaning until necropsy. The eyes of vehicle control, reference control and high dose LNT animals were examined in Week 13. Blood samples were taken for haematology, blood chemistry and coagulation during Week 13 and at the end of the treatment-free period. Additional blood samples were taken at the end of the treatment period for potential analysis of thyroid stimulating hormone (TSH), T3 and T4 and were stored frozen until the end of the study. Urine samples were collected for urinalysis in Week 13 and at the end of the treatment-free period.

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

All surviving animals (at the end of the treatment and recovery periods) were subjected to a gross macroscopic necropsy, selected organs/tissues were weighed and fixed. At the end of the treatment period, a full list of organs/tissues for early decedents and animals in the vehicle control, high-dose LNT and reference control groups, were examined microscopically.

There were no test item-related deaths. Six animals (1 male and 1 female from the vehicle control group, 1 male and 1 female given 1,000 mg/kg body weight/day LNT, 1 reference control female and 1 female given 4,000 mg/kg body weight/day LNT) died during the study, but macro- and microscopic examinations did not identify any test item-related lesions in these animals. Three of the deaths were considered to be due to dosing trauma and there was no specific cause of death reported for the other 3 decedents, but all were considered incidental and unrelated to treatment with LNT.

No test item-related clinical signs or ocular findings were observed. Animals administered LNT gained similar amounts of weight and consumed similar amounts of food compared with controls. Overall mean food intake for the high-dose males was statistically significantly lower than vehicle controls, but the value (23 g/animal/day) was exactly the same as reported for males provided LNT at a dose of 1,000 mg/kg body weight/day or the reference controls over the same period (for both of which statistical significance was not observed) and was therefore considered to be unrelated to LNT administration.

LNT administration had no effect on pre-weaning development [as evaluated by the age of attainment of the surface and air righting reflexes, and the pupil reflex and startle response tests conducted on Day 14 of treatment (Day 20 of age)]. Ulna length and growth were similar between LNT-treated groups and controls. No test item-related differences in behaviour of the animals during the in-hand and arena observations in Week 11 of treatment (Day 81 to 83 of age) were observed. Morris maze performance was also unaffected by administration of LNT, with clear evidence of learning and memory over the 4 days of testing, as demonstrated by generally progressive decreases in group mean trial times, sector entries and failed trials.

There were no test item-related differences for the mean body weight or day of age at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation and vaginal opening for males and females, respectively). The mean body weight at attainment of sexual maturation for females given 2,500 or 4,000 mg/kg body weight/day LNT was statistically significantly higher than for vehicle controls. However, the values were similar to those of reference controls and the differences from vehicle controls were minor, with no dose-response relationship observed.

No test item-related differences in values for haematological parameters between LNT-treated groups and vehicle controls were observed. At the end of the treatment period, statistically significantly increased mean cell haemoglobin (for males given 4,000 mg/kg body weight/day LNT) and mean cell haemoglobin concentration (for both sexes given 4,000 mg/kg body weight/day LNT) compared with vehicle controls were observed, but the differences were minor and values were similar to those for reference controls. Statistically significant reductions in red cell distribution width (for females given 2,500 or 4,000 mg/kg body weight/day LNT), mean cell volume and haematocrit (for females given 4,000 mg/kg body weight/day LNT) were not associated with a dose response, similar changes were not seen for males and the values were similar to those seen for reference controls. The majority of individual values for all of these parameters were also within the respective historical control data (HCD) ranges.

A statistically significant increase in leukocytes for males dosed at 4,000 mg/kg body weight/day LNT was a result of increased lymphocytes, eosinophils and large unstained cells, which was not observed for female groups (values for the female high-dose LNT group were lower or equal to those of vehicle controls for the same parameters). These differences were considered to be due to vehicle control males having atypically low leukocyte values, as evidenced by the historical control ranges for all of these parameters: 5 of the 10 vehicle control males had values below the HCD lower limit for animals of the same strain and age (8.77 x 10^9/L). The mean value for this group (8.70 x 10^9/L) was also notably lower than the HCD mean (14.21 x 10^9/L) and below the HCD lower limit for individuals. In comparison, only 4, 3 and 2 individual values for males given 1,000, 2,500 or 4,000 mg/kg body weight/day LNT were below the HCD lower limit and the mean value for high-dose males (12.67 x 10^9/L) was the nearest of any group to the HCD mean. Similarly for

lymphocytes and large unstained cells (LUC), 3 and 5 out of 10 individual male vehicle control values, respectively, were below the HCD lower limit, whereas for high-dose males, all individual values and 9 of the 10 LUC values were within the HCD ranges for these parameters; mean values for high-dose males (10.81 and 0.08 x 10^9/L for lymphocytes and LUC, respectively) were very similar to the respective HCD means (10.72 and 0.11 x 10^9/L for lymphocytes and LUC, respectively), as opposed to vehicle control means (7.13 and 0.05 x 10^9/L for lymphocytes and LUC, respectively), which were notably lower than the HCD values. Statistically significant changes among females were limited to a decreased neutrophil concentration at 4,000 mg/kg body weight/day LNT, which again was inconsistent between the sexes as it was not seen for males and all but 1 of the individual values for this group were within the HCD range. Further evidence that these differences were clearly not test-item related include the lack of any associated changes that would usually correlate with effects on immune function; for example, there were no differences in rectal temperature as assessed in the in-hand observations, no test item-related differences in spleen weights nor any macroscopic or microscopic findings related to LNT administration.

Platelets were statistically significantly increased for males given 4,000 mg/kg body weight/day LNT compared with vehicle controls, but there was no dose response relationship nor any consistency between the sexes. There were also no biologically relevant differences in the other clotting parameters [prothrombin time (PT) and activated partial thromboplastin time (APTT)] for LNT-treated groups compared with controls.

No test item-related differences in values for blood chemistry parameters between LNT-treated groups and vehicle controls were observed. Where statistically significant differences compared with vehicle controls were observed, there was either no dose-response relationship or the differences were inconsistent between the sexes. For all parameters, high dose values were comparable with those of reference controls and individual values for all LNT treated groups were generally within HCD ranges. In the absence of any test item related effects in the treatment period, minor statistically significant differences between LNT-treated groups and vehicle controls observed during the recovery period were considered biologically irrelevant and unrelated to LNT administration.

There were no test item-related differences in urinalysis parameters between LNT-treated groups and controls. For females, a statistically significant increase in urine volume (at 4,000 mg/kg body weight/day) and statistically significantly reduced specific gravity for all LNT-treated female groups were observed at the end of the treatment period compared with vehicle controls. There were no statistically significant differences in urine volume for LNT-treated males, but specific gravity was statistically significantly lower than vehicle controls for males given 2,500 or 4,000 mg/kg body weight/day LNT. These findings were considered biologically irrelevant and unrelated to LNT administration as all individual values for these parameters were within the HCD ranges for animals of this age and strain, indicating the values were within normal biological variation; there was also no evidence of an alteration in kidney function; there were no biologically relevant differences in total protein (statistically significant differences for males were not associated with a dose response), creatinine or glucose concentrations between LNT-treated groups and controls, nor were there any microscopic abnormalities in urine sediment; and no test item-related differences in associated blood chemistry parameters, kidney weights or in the incidences of macroscopic or microscopic kidney findings were observed.

Organ weights were unaffected by LNT administration. The only statistically significant differences in body weight-relative organ weights observed in LNT-treated groups compared to vehicle controls at the end of the treatment period were increases in testes weights for males given 2,500 or 4,000 mg/kg body weight/day LNT, but there was no evidence of a dose response. Statistically significantly increased body weight-relative kidney, liver, ovary and spleen weights for females given 4,000 mg/kg body weight/day LNT

were only seen at the end of the treatment-free period and were not evident immediately after cessation of dosing, indicating that these changes were unrelated to treatment with LNT. As there was no effect on the pituitary-thyroid axis observed during the study, the samples collected for potential analysis of TSH, T3 and T4 were not analysed; this is in accordance with OECD Test Guideline 407 (OECD, 2008), which the EFSA *Guidance for submission for food additive evaluations* refers to regarding modification of OECD Test Guideline 408 (OECD, 1998b) studies, to include assessment of some additional parameters that place more emphasis on endocrine-related endpoints (EFSA, 2013).

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

In absence of any test item-related adverse effects, the NOAEL was concluded to be 4,000 mg/kg body weight/day LNT (the highest dose tested and maximum feasible dose, limited by viscosity of the test article).

6.4.1.2 Genotoxicity Studies

6.4.1.2.1 Bacterial Reverse Mutation Test

The potential mutagenicity of LNT was evaluated in a bacterial reverse mutation test (Ames test), which was performed in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 471 (OECD, 1997), Commission Regulation (EC) No 440/2008[13] B13/14, U.S. EPA Health Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998) and FDA Redbook IV.C.1.a. (U.S. FDA, 2000) (Phipps *et al.*, 2018).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium (*S.* Typhimurium) strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* (*E. coli*) strain WP2 uvrA (pKM101), which were treated with LNT at concentrations of up to 5,106.1 µg/plate (due to a minor error in the application of the correction factor, the high concentration slightly exceeded the intended high concentration of 5,000 µg/plate — the OECD 471 guideline maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix).

Water (purified by reverse osmosis) served as the vehicle for LNT and as the negative control. Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide) and presence [2-aminoanthracene and benzo(a)pyrene in the presence of metabolic activation] of metabolic activation. A positive result for mutagenicity was defined as a dose-dependent and biologically relevant 2- or 3-fold increase in the number of revertant colonies, compared to that of the vehicle control group.

There was no evidence of mutagenicity following exposure to LNT in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in revertant colony counts (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that LNT is non-mutagenic at concentrations up to 5,106.1 µg/plate (slightly above the regulatory maximum dose level).

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

6.4.1.2.2 *In Vitro* Mammalian Cell Micronucleus Test

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

An initial preliminary cytotoxicity test was conducted using LNT at concentrations up to 2,042.44 µg/mL[14], in the presence (3-hour treatment) and absence (3 and 24-hour treatments) of S9 metabolic activation; no cytotoxicity was observed at any dose level. Cytotoxicity was assessed again in the main experiment and again there was no evidence of cytotoxicity at any dose level under any of the experimental conditions.

In the main experiment for micronucleus analysis, human lymphocytes were treated with concentrations of LNT at 510.61, 1,021.22 or 2,042.44 µg/mL with S9 (3 hours) and without S9 (3 and 24-hour treatments). The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronucleated binucleated cells (MNBC), with the frequency of MNBC also being above upper historical vehicle control limit.

There was no evidence of clastogenicity or aneugenicity in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in MNBC (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that LNT is neither clastogenic nor aneugenic at concentrations up to 2,042.44 µg/mL, in the absence and presence of metabolic activation.

6.4.2 Studies Conducted with LNnT

As mentioned previously, LNnT is a chemically and structurally similar isomer of LNT. Therefore, the toxicological data obtained for LNnT preparations are relevant to the safety evaluation of LNT. The studies conducted with Glycom's LNnT obtained by chemical synthesis and microbial fermentation have been previously reviewed by various qualified Experts including the U.S. FDA and EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) (GRN 547, 659 EFSA NDA Panel, 2015a,b). The evaluations have consistently concluded that LNnT is safe for its intended uses. These studies are incorporated by reference to previous GRAS notifications and are described in brief below.

6.4.2.1 Repeat-Dose Oral Toxicity

The subchronic toxicity of LNnT produced by chemical synthesis by Glycom has evaluated in a subchronic (90-day) oral toxicity study where neonatal Wistar [Crl:WI(Han)] rats were administered LNnT by gavage at doses of 0 (water vehicle control), 1,000, 2,500 or 5,000 mg/kg body weight/day, from post-natal day (PND) 7 until 13 weeks of age (Coulet *et al.*, 2013). The study was conducted in accordance with OECD principles of GLP (OECD, 1998a) and OECD Test Guideline 408 (OECD, 1998b), with an adaptation to include the use of neonatal rats. In their evaluation of this study, the EFSA NDA Panel (EFSA NDA Panel, 2015a) concluded the following:

> *"Based on the observations on reticulocytes, platelet counts, Hb [haemoglobin] levels and PCV [packed cell volume] in the high-dose LNnT group (5,000 mg/kg body weight per day)*

[14] Due to a minor error in the application of the correction factor, the high concentration slightly exceeded the intended high concentration of 2,000 µg/mL—the OECD 487 guideline maximum recommended concentration

and the decrease in the zymogen content in acinar cells in three animals in the high-dose LNnT group, the Panel considers that the no observed adverse effect level (NOAEL) is 2,500 mg/kg body weight per day".

However, these statements are not consistent with conclusions of other independent Experts (*e.g.*, in previous GRAS Panel evaluations of LNnT), and the apparent changes reported by the NDA Panel have not been reported in subsequent subchronic toxicity evaluations of LNnT (or LNT) conducted in neonatal rats at doses up to 5,000 mg/kg body weight/day (Penard, 2016; Phipps *et al.*, 2018). Accordingly, the original NOAEL determination by Coulet *et al.*, (2013) of 5,000 mg/kg body weight/day is considered an appropriate conclusion.

Prieto (2005) reported findings from a subacute 28-day toxicity study of LNnT in rats. The LNnT test article investigated in these studies was produced using a glycosyltransferase derived from yeast. In the 28-day study, 12 litters (5/sex/litter) of Cri:CD®(SD)BR rat pups (15 days old) were administered 0 (control), 10, 200, or 400 mg/kg body weight/day of LNnT (purity not reported) *via* gavage. Parameters evaluated included urinalysis, haematology, faecal analysis and gross pathology. There were no significant differences in any of the parameters measured reported among groups. In the dietary study, 31- to 37-day-old rats (sex, strain, and number not reported) were fed diets containing 1 or 5% of LNnT for a period of 4 weeks[15]. The dietary levels of LNnT provided are equivalent to a dose of approximately 1,000 and 5,000 mg/kg body weight/day, respectively, based on an average body weight of 100 g reported for young rats (U.S. FDA, 1993). It is unknown whether a control group was included in this study. Detailed clinical chemistry and histopathological examinations were conducted (no further details were provided). No test article-related adverse effects or macroscopic and microscopic changes were observed following LNnT administration. No further details on these studies were provided in the publication by Prieto (2005) although the publication provides a general indication that LNnT lacks toxicity in rats at the doses administered.

The oral toxicity of LNnT produced by fermentation (94.4% LNnT by assay) was investigated in an adapted subchronic (90-day) oral toxicity study in 7-day-old Wistar [Crl:WI(Han)] rats. The study was conducted in accordance with the OECD Principles of GLP (OECD, 1998a), OECD Test Guideline No. 408 (OECD, 1998b) with an adaptation to include the use of neonatal rats (Penard, 2016). For a comprehensive summary of this study the reader is directed to Section IV.E.1 and Appendix A of GRN 659. The studies were conducted in neonatal rats starting on PND 7 to adequately cover the developmental window during which infants will be exposed. Fructo-oligosaccharide (FOS) was used as the reference control on the basis that it is currently authorised as an ingredient in infant and follow-on formulae. Rats (10/sex/group) were orally administered by gavage at doses of 0 (water vehicle control), 1,000, 2,500 or 5,000 mg/kg body weight/day of LNnT or FOS at 5,000 mg/kg body weight/day, for at least 90 days. Additional groups of 5 males and 5 females were given the control, high-dose LNnT, or FOS for the same period of 90 days, after which they were kept un-dosed for a 28-day treatment-free period. A NOAEL of 5,000 mg/kg body weight/day, the highest dose tested, was established based on the results of this study.

6.4.2.2 Genotoxicity Studies

Published genotoxicity studies conducted with LNnT obtained by chemical synthesis, include a bacterial reverse mutation assay, an *in vitro* mammalian cell gene mutation test and an *in vitro* mammalian cell micronucleus test. Details of the bacterial reverse mutation assay and *in vitro* mammalian cell gene mutation test have been published by Coulet *et al.* (2013), while the *in vitro mammalian cell micronucleus*

[15] There is some ambiguity with respect to the duration of this study that was reported by Prieto (2005). In the *Materials and Methods* section of this publication, it was indicated that these rats were administered diets containing LNnT for 4 weeks. However, within the *Results* section, the dietary experiment was referred to as being 4 months in duration.

test is described in Section IV.B.5.3 of GRN 547. These studies were all conducted in accordance with OECD test guidelines. Critical evaluations of these studies by previous GRAS Panel's and the EFSA NDA Panel have concluded that LNnT does not pose any safety concerns with respect to genotoxicity (GRN 547, GRN 659, EFSA NDA Panel, 2015a).

The mutagenicity of LNnT produced by fermentation (94.4% LNnT by assay) was evaluated in a bacterial reverse mutation assay in *S.* Typhimurium strains TA98, TA100, TA1535, and TA1537 and in *E. coli* strain WP2uvrA in the presence or absence of metabolic activation (S9), using the plate incorporation and pre-incubation methods (See Section IV.E.2 of GRN 659). The study was conducted in accordance with the OECD principles of GLP and according to OECD Test Guideline No. 471 (OECD, 1997, 1998a). Using the plate incorporation method, bacterial strains were treated with LNnT at concentrations of 52, 164, 512, 1,600, or 5,000 µg/plate in the presence or absence of S9. For the pre-incubation method, bacterial strains also were incubated with LNnT at concentrations of 52, 164, 512, 1,600, or 5,000 µg/plate in the presence or absence of S9. LNnT was determined to be non-mutagenic under the conditions of the bacterial reverse mutation assay in the presence or absence of exogenous metabolic activation at concentrations up to 5,000 µg/plate.

The genotoxicity of LNnT produced by fermentation (94.4% LNnT by assay) was further investigated in an *in vitro* micronucleus assay conducted in cultured peripheral human lymphocytes (See Section IV.E.2 of GRN 659). This study also was conducted in compliance with the OECD principles of GLP and according to OECD Test Guideline No. 487 (OECD, 1998a, 2014). Mitomycin C and colchicine were used as the positive controls in the absence of metabolic activation (S9 mix) and cyclophosphamide was used as the positive control in the presence of S9 mix. Water was used as the negative control. In the short-term exposure experiment, lymphocytes were incubated with LNnT at concentrations of 512, 1,600, or 2,000 µg/mL for 3 hours in the presence or absence of S9, following which the cells were rinsed and incubated for another 24 hours prior to scoring. In the long-term exposure experiment, cells were treated with LNnT at concentrations of 512, 1,600, or 2,000 µg/mL for 24 hours in the absence of S9. At least 1,000 binucleated cells and 1,000 mononucleated were scored for micronuclei under each treatment condition. LNnT was determined to be non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.

6.5 Human Studies

6.5.1 Clinical Studies on LNT

No human studies have been conducted using LNT; however, human data for the structural isomer LNnT have been published and are discussed below in Section 6.5.2.

6.5.2 Clinical Studies on LNnT

Two clinical studies in infants evaluating the safety and tolerance of LNnT have been published (Prieto, 2005; Puccio *et al.*, 2017).

The study by Prieto *et al.* (2005) was conducted using 228 healthy male and female infants and toddlers between the ages of 6 to 24 months attending day care centers of the Junta Nacional De Jardines Infantiles of Santiago De Chile (Chile) who were provided infant formula supplemented with LNnT at a use level of 220 mg/L. Infants in the control group received the same formula without LNnT. Formulas were provided for 112 days and study endpoints included measures of oropharyngeal colonization rate *of S. pneumonia*, otitis media and related adverse ear pathologies, formula intake, and body weight and length. Average daily consumption of the control formula ranged from 511 to 602 mL/infant/day and average daily consumption

of the LNnT supplemented formula ranged from 506 to 559 mL/ infant/day (no statistically significant difference in overall formula intake between groups). Based on the results of the study, LNnT produced using a yeast fermentation process was well-tolerated in infants and was without adverse effects on growth and ear health at a concentration of 220 mg/L.

The study by Puccio *et al.* (2017) was a randomised, double-blinded, controlled, multi-centre, parallel-design study where healthy, full-term infants were provided a standard term infant formula supplemented with LNnT (providing 0.5 to 0.6 g LNnT/L reconstituted formula) in combination with 2'-FL (providing 1.0 to 1.2 g 2'-FL/L of reconstituted formula). A comparator group receiving a standard whey-predominant starter infant formula without HiMOs was included as a control. Infants were aged 0 to 14 days at enrolment, and clinic visits were scheduled at 1, 2, 3, 4, 6, and 12 months of age. The infants were exclusively fed the test or control formulas for the first 4 months of age, with complementary foods allowed to be introduced thereafter. At 6 months of age, the infants in both study groups (test and control formula) were switched to an intact protein, cow milk–based follow-up formula without HiMOs for feeding through to 12 months of age. Endpoints related to growth (body weight gain, body weight, body length, and head circumference), as well as gastrointestinal tolerance, behavioural patterns, and morbidity were collected through to 12 months of age. Additionally, the effects of LNnT supplementation on the intestinal microbiota profile were also assessed in the infants (Alliet *et al.*, 2016; Steenhout *et al.*, 2016). The study by Puccio *et al.* (2017) was previously evaluated during by the LNnT GRAS Panel (See IV.F.1 of GRN 659), and also was evaluated the EFSA NDA Panel (EFSA NDA Panel, 2015a) during their novel food evaluation of LNnT. The NDA Panel reported the following conclusion:

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

The safety and tolerability of LNnT was also investigated in a randomised, placebo-controlled, double-blind, parallel-design study involving healthy adult volunteers (Elison *et al.*, 2016). The participants were provided either LNnT or 2'-FL alone (at doses of 5, 10, or 20 g per day of LNnT or 2'-FL), or a combination of LNnT and 2'-FL (5, 10, or 20 g per day as the combined amount of LNnT and 2'-FL at a ratio of 2:1). A comparator group received glucose only as a placebo control. All test articles were consumed as single daily bolus doses for 2 weeks. LNnT was generally well tolerated in the study. The EFSA NDA Panel recognised that when compared to the placebo control, consumption of either 10 or 20 g of LNnT resulted in a significant increase in passing gas, while, for subjects consuming 5 g per day, this effect was not observed (EFSA NDA Panel, 2015a). However, these effects were minor, generally being rated at a discomfort level of "mild" or less and consumption of such high levels of bolus intake are not realistic under the proposed conditions of use for LNnT in foods.

6.6 Allergenicity

As explained in Section 2.3.3.2, the high purification steps involved in the manufacture are proven to remove protein (*i.e.*, potential allergen) to a level of <0.0017% (w/w). Glycom's supplier management procedure ensures that all suppliers of raw materials and processing aids must declare and demonstrate that their materials do not contain any of the allergens specified under Regulation (EU) No 1169/2011 of the

European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers[16].

In addition, Glycom have assessed allergenic potential of the recombinant proteins using the search algorithms provided by the Allergen Online tool (ver. 18B) hosted by the University of Nebraska (FARRP, 2018). This database has been updated last on 23 March 2018 and contains sequences of 2,089 putative allergens. The online tool allows search by 3 different search algorithms each with its own alert limit for potential allergenicity: (i) Full sequence length (FASTA) comparison with an alert limit of min. 50% sequence similarity to hint for potential allergenic potential (ii) 80 amino acid sequence segments (sliding window) comparison with an alert limit of min. 35% sequence similarity to hint for potential allergenic potential (iii) 8 mer sequence segments (sliding window) with an alert limit of full match to hint for potential allergenic potential. No sequence alerts for potential allergenicity were identified.

6.7 Expert Panel Evaluation

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

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

6.8 Conclusion

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

LNT 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.

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

Part 7. § 170.255 List of Supporting Data and Information

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Expert Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of Lacto-*N*-tetraose (LNT) for Uses in Infant Formula and Conventional Food Products

06 November 2018

INTRODUCTION

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

The Expert Panel, independently and collectively, critically evaluated a comprehensive package of publicly available scientific information and data compiled from the literature and presented to the Expert Panel in a dossier titled "*GRAS Status of Lacto-N-tetraose (LNT)*" (dated 18 October 2018), which included an evaluation of all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food uses of LNT. This information was prepared in part from a comprehensive search of the scientific literature performed by Glycom and included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, the history of consumption from human breast milk, and the safety of LNT.

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

SUMMARY AND BASIS FOR GRAS

Glycom A/S (Glycom), a manufacturer of HiMOs, intends to introduce an LNT preparation consisting primarily of LNT (min. 70%), and lesser levels of lactose (max. 12%) and lacto-*N*-triose II (max. 10%) to the United States (U.S.) marketplace as a food ingredient for addition to non-exempt term infant formula and various conventional food and beverage products (Table A-1). The LNT preparation is produced by fermentation using a modified strain of *E. coli* K-12 and contains no less than 90% of human-identical milk saccharides (HiMS), which is characterized by the sum of LNT, lactose, and lacto-*N*-triose II in the final

product. LNT is a neutral tetrasaccharide derived from lactose by subsequent addition of *N*-acetylglucosamine (GlcNAc) and galactose in linkage-specific manner. The mean concentration of LNT in pooled human milk is higher than that of all other neutral human milk oligosaccharides (HMO). Glycom conducted comprehensive reviews of the published literature for studies evaluating the concentration of HiMOs in human milk. Based on these studies, Glycom concluded that, globally, the estimated range of LNT in mature human breast milk ranges from 0.1 to 3.7 g/L, and an average mean concentration of 0.9±0.4 g/L was estimated. LNT produced by microbial fermentation is chemically and structurally identical to LNT that is naturally present in human breast milk, as confirmed by 1H- and 2D-nuclear magnetic resonance-spectroscopy and mass spectrometry. Therefore, LNT has an established long history of safe consumption as a component of human breast milk in infants on the basis that LNT manufactured by Glycom is chemically identical to LNT naturally present in human breast milk.

The Expert Panel critically reviewed details of the manufacturing process for LNT. The ingredient is manufactured in compliance with cGMP and incorporates a Hazard Analysis Critical Control Point (HACCP) management system. The manufacturing process can be broadly divided into 2 stages. In Stage 1 (upstream processing), D-lactose is converted to LNT by a sequential series of enzymatic steps using the adapted cellular metabolism of the production microorganism, which uses D-glucose (or optionally D-glycerol or D-sucrose) as an exclusive energy and carbon source and D-lactose as a substrate for LNT biosynthesis. The production microorganism is a derivative of *E. coli* K-12 DH1, which is a non-pathogenic laboratory strain with a well-characterized genetic history (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996). *E. coli* K-12 DH1 was optimized for general oligosaccharide expression features by the introduction of several modification events related to the metabolism of various carbohydrates (identical to those described in GRAS Notice [GRN] 650). The strain was genetically modified to genomically and episomally overexpress the β-1,3-*N*-acetylglucosaminyl-transferase gene from *Neisseria meningitidis* and β-1,3-galactosyl-transferase gene from *Helicobacter pylori* required for LNT biosynthesis. Two key native genes encoding for quinolinate phosphoribosyl-transferase were deleted from the genome and provided to the cells *via* plasmid in order to tightly link cell survival and LNT production. No antibiotic resistance genes are present, and antibiotics and inducer molecules are not used. The Expert Panel noted that the identities of the introduced genes and their expression products (*i.e.*, enzymes) are well characterized, and the introduced genes would not confer toxicogenic/pathogenic properties to the host organism. The recombinant proteins were further characterized using bioinformatic tools and are not homologous to amino acid sequences of known or putative toxins or allergens.

In Stage 2 (downstream processing), a series of purification, isolation, and concentration steps are used to generate the final high-purity LNT ingredient. No solvents are used during manufacturing and Glycom confirms that all processing-aids and food contact articles used in Stage 1 and 2 are used in accordance with an appropriate federal regulation, have been previously determined to be GRAS or have been the subject of an effective food contact notification. Quality control measures are in place during the entire purification and isolation process to ensure that the final batches of LNT released conform with the product specifications. The LNT produced by fermentation is chemically identical to the LNT present in human milk from lactating women. There have been no modifications to the molecular structure of LNT during the manufacturing process from that of LNT that is present in human milk.

Glycom has established food-grade specifications for LNT. The specifications for LNT include parameters related to physical properties, purity, water, ash content, and microbiological contaminants. The main components of the ingredients are comprised of a simple HiMS mixture predominantly consisting of LNT (min. 70%), with some levels of lactose (max. 12%) and lacto-*N*-triose II (max. 10%). Specification limits for

possible by-products and degradation[1] products such as *para*-lacto-*N*-hexaose-2 (max. 3.5%) and the LNT fructose isomer[2] (max. 1%) have been established. As lactose and lacto-*N*-triose II are also naturally present in human milk, the total human milk saccharide content of the ingredient is at least 90%. Specifications have been established for carbohydrate-type compounds and residual proteins originating from the fermentation and downstream purification processes. All analytical methods are internationally recognized or have been validated internally. The Expert Panel reviewed the results from 5 batches of LNT demonstrating that the manufacturing process produces a consistent material in conformance with the product specifications.

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

The Expert Panel reviewed the data supporting the bulk stability of LNT, produced by fermentation as described herein, under real-time conditions of 25°C and 60% relative humidity (RH), as well as accelerated conditions of 40°C and 75% RH, over a 24- and 9-month storage period, respectively. LNT was stable throughout the storage period with no appreciable changes in organoleptic properties, degradation of the material, or alterations in the impurity profile. Stress/forced stability tests of amorphous and crystalline LNT powder in aqueous solutions were performed at 60°C and 80°C for 8 and 4 weeks of storage, respectively, to identify potential degradation products of LNT under slightly acidic pH (4.5) and neutral pH (6.3 to 6.8). The results of these studies showed 2 potential pH-dependent chemical degradation pathways of LNT, namely hydrolysis at pH <5.0 to generate glucose and lacto-*N*-triose II and isomerization at pH >6.0 to generate LNT fructose isomer. The stability of LNT described herein has also been evaluated in a commercially-representative infant formula, with data supporting that LNT is stable in infant formula at up to 12 months of storage.

Glycom provided a summary on the stability of lacto-*N*-neotetraose (LNnT) in various food matrices, such as yogurts, ready-to-drink flavored milk, and citrus fruit beverages. Owing to the chemical and structural similarities of LNT to LNnT, there are no anticipated differences in their stabilities in food matrices. LNT has been demonstrated to be stable in infant formula and representative food and beverages under the conditions of these studies.

LNT is intended to be added as a food ingredient to foods targeted to infants and young children, including non-exempt term infant formula, and in specific conventional food products used by the general population (Table A-1). The maximum use-levels in term infant formulas are proposed on the basis of providing similar levels of LNT on a body weight basis as those consumed by breast-fed infants. In the U.S. food uses of LNT in infant formula (*i.e.*, infants up to 12 months) will provide LNT at a use-level of 0.8 g/L, follow-on formula at a use-level of 0.6 g/L, infant-specific foods and foods for young children at a use-level of 0.6 g/L in ready-to-drink and reconstituted products, and up to 5 g/kg for products other than beverages (*e.g.*, baby foods). LNT is also intended for use in food and beverages targeted towards the general U.S. population (up to 1.0 g/L or 10 g/kg), and foods for special dietary use (*e.g.*, meal replacement bars) at levels up to 2.0 g/L or 20 g/kg.

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[1] It should be noted that lactose and lacto-*N*-triose II are also principal degradation products of LNT.

[2] "LNT fructose isomer" denotes an isomer of LNT where the terminal glucose sugar isomerised to a fructose sugar.

The Expert Panel reviewed data related to the estimated dietary exposure to LNT based on an assessment of the anticipated intake of LNT as an ingredient under the intended conditions of use provided by Glycom. This information included data on the natural presence of LNT in human breast milk (colostrum, transitional milk, mature milk) from various countries, which demonstrates the established history of safe consumption of LNT by infants consuming human milk. Differences in the levels of LNT in human breast milk have been reported for different countries, and a wide interindividual variation in concentrations of LNT is apparent between individual mothers (*i.e.*, "Secretors" and "Non-secretors"). Based on studies identified in the literature, Glycom has estimated that an average mean concentration of *ca.* 1 g/L of LNT is reflective of typical levels that have been measured across most studies. Based on the proposed use levels of LNT described in Table A-1, and using information from the 2013-2014 cycle of the National Health and Nutrition Examination Survey (NHANES), an estimation of probable consumption levels of LNT from all food uses was obtained. On a consumer-only basis, the resulting mean and 90th percentile intakes of LNT by the total U.S. population from all proposed food-uses, were estimated to be 0.83 g/person/day (17.2 mg/kg body weight/day) and 1.77 g/person/day (33.6 mg/kg body weight/day), respectively. Among the individual population groups, the highest mean and 90th percentile consumer-only intakes of LNT were determined to be 0.94 g/person/day (11.0 mg/kg body weight/day) and 2.02 g/person/day (24.9 mg/kg body weight/day), respectively, among male adults. The elderly had the lowest mean consumer-only intakes of 0.66 g/person/day (8.8 mg/kg body weight/day), while female teenagers had the lowest 90th percentile consumer-only intakes of 1.37 g/person/day (26.4 mg/kg body weight/day). When intakes were expressed on a body weight basis, toddlers had the highest mean and 90th percentile consumer-only intake of 70.2 and 141.0 mg/kg body weight/day.

The Expert Panel critically evaluated the published data and information characterizing the safety of LNT. Due to the fact that LNT is chemically and structurally identical to its naturally occurring counterpart in human milk, LNnT, published safety data and information supporting the GRAS status of LNnT that have been discussed previously (*i.e.*, GRN 547 and GRN 659) were considered relevant to the safety evaluation of LNT. The Expert Panel noted that all HiMOs manufactured by Glycom are produced from a production organism originating from the same MDO lineage strain and that all HiMOs are produced by Glycom are based on fermentation processes that utilize lactose as substrate, and defined carbon and nitrogen sources. Toxicology studies conducted for Glycom's existing portfolio of HiMOs produced by fermentation (*e.g.*, 2'-fucosyllactose [2'-FL], LNnT) support the safety of the MDO lineage. The introduced genetic modifications for HiMO synthesis produce a predictable pattern of metabolites and intended fermentation products that are identifiable and are not of concern for imparting unexpected pleiotropic effects to fermentation products produced from this host. The Expert Panel noted that there have been no toxicologically significant findings in any reported toxicity study that suggests that 2'-FL, or other HiMOs produced using Glycom's MDO strain lineage, display toxicity profiles in animals that would be of concern for use as an ingredient in formula or other conventional food products. The results of product specific toxicology studies conducted with LNT as discussed below further support this safety conclusion.

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

The Expert Panel evaluated the results of a 14-day toxicity study and a 90-day toxicity study conducted in neonatal Crl:CD(SD) rats administered LNT manufactured by Glycom at doses up to 4,000 mg/kg body weight/day (Phipps *et al.*, 2018). No test article-related adverse findings were reported in the 14-day toxicity study, which served as the basis for the tested doses in the subsequent 90-day toxicity study. The 90-day toxicity study was conducted in accordance with the Organisation for Economic and Co-operation Development (OECD) Principles of Good Laboratory Practices (GLP) and OECD Test Guideline 408 modified for the target population (*i.e.*, infants) considering the requirements of the European Food Safety Authority (EFSA) Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (EFSA, 2017), Guidance for industry: nonclinical safety evaluation of paediatric drug products (U.S. FDA, 2006), Guideline on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications (EMEA, 2008), and the Guideline on the Nonclinical Safety Study in Juvenile Animals for Paediatric Drugs (MHLW, 2018). In the 90-day toxicity study, neonatal Crl:CD(SD) rats (10/sex/group) were administered LNT at doses of 0, 1,000, 2,500, or 4,000 mg/kg body weight/day and were examined for the standard toxicological battery (mortality, clinical signs, hematology, clinical chemistry, urinalysis, macroscopic and microscopic examination). No test article-related adverse findings were reported in any study parameter at doses up to the highest dose tested (*i.e.*, 4,000 mg/kg body weight/day, the maximum feasible dose based on viscosity), and therefore, the no-observed-adverse-effect level (NOAEL) was concluded to be 4,000 mg/kg body weight/day.

The Expert Panel also reviewed the results of genotoxicity and mutagenicity bioassays conducted with Glycom's LNT. The potential mutagenicity of LNT was evaluated in the bacterial reverse mutation test using *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were treated with LNT at concentrations of up to 5,106.1 µg/plate in the presence and absence of metabolic activation. The potential genotoxicity of LNT was evaluated in an *in vitro* mammalian cell micronucleus test conducted using human lymphocytes at concentrations of LNT at 510.61, 1,021.22, or 2,042.44 µg/mL with S9 (3 hours) and without S9 (3 and 24-hour treatments). Both bioassays were performed in accordance with respective OECD Test Guidelines and Principles of GLP. The results of these studies confirm that LNT, like other HMOs that are natural constituents of human breast milk, are non-mutagenic, non-clastogenic, and non-aneugenic, and that the manufacturing process did not introduce undesirable substances with potential mutagenic/genotoxic potential.

In addition to the studies on LNT, studies evaluating the subchronic toxicity of LNnT preparations produced by chemical synthesis or fermentation were conducted in neonatal rat models and *in vitro* and *in vivo* genotoxicity studies; these studies were reviewed by various qualified Experts during previous GRAS evaluations and by the U.S. Food and Drug Administration (GRN 547 and GRN 659) and EFSA. The results of animal toxicity studies consistently demonstrated that LNnT is without evidence of toxicity up to the highest dose tested, findings that are consistent with the safe history of consumption of LNnT by breast-fed infants. Results of multiple genotoxicity batteries, including the Ames reverse mutation assay, *in vitro* chromosomal aberration assays, mouse lymphoma cell gene mutation test, and *in vitro/in vivo* micronucleus assays were all negative for evidence of genotoxicity/mutagenicity. The Expert Panel noted that due to the chemical and structural similarity between LNT and LNnT, the safety conclusions of the studies conducted with LNnT can be extended to support the safety of LNT.

Studies evaluating the safety and tolerance of LNnT in infant formula were previously evaluated in GRN 659 (Prieto *et al.*, 2005; Puccio *et al.*, 2017). Healthy, full-term infants were provided formula supplemented with 0.5 to 0.6 g LNnT (in combination with 1.0 to 1.2 g 2'-FL) for the first 4 months of life and were shown to be well-tolerated and supported age-appropriate growth (Puccio *et al.*, 2017). In adults, the results of a safety and tolerability study indicate that consumption of LNnT at doses of up to 20 g, 2'-FL at doses of up to 20 g, or their combination at a 2:1 ratio (for total sum of up to 20 g), when taken as a single bolus dose daily

for 2 weeks, was well-tolerated and did not result in any deviations in laboratory measures of safety (*i.e.*, hematology and blood biochemistry) compared to normal reference values (Elison *et al.*, 2016). Details of 2 of these studies (Elison *et al.*, 2016; Puccio *et al.*, 2017) were incorporated by reference to GRN 546 and 650. The Expert Panel noted that all these studies were previously reviewed by Expert Panels during previous GRAS evaluations and by other qualified experts including the EFSA Panel on Dietetic Products, Nutrition and Allergies Panel during the Novel Food evaluation of 2'-FL (EFSA, 2015). Findings from these studies consistently demonstrated that administration of HiMOs at concentrations that are representative of levels in human breast milk is safe and well tolerated.

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

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

CONCLUSION

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

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

[redacted]

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

12 November 2018
Date

[redacted]

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

20 November 2018
Date

[redacted]

Professor Emeritus Robert J. Nicolosi, Ph.D.
University of Massachusetts Lowell

15 November 2018
Date

[redacted]

Professor Emeritus John A. Thomas, Ph.D.
Indiana University School of Medicine

18 Nov. 2018
Date

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ATTACHMENT A1: INTENDED FOOD USES AND USE-LEVELS FOR LNT IN THE UNITED STATES

Table A-1 Summary of the Individual Proposed Food Uses and Use-Levels for LNT in the U.S.

Food Category	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use-Level (g/RACC)	Proposed Maximum Use-Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction[b]	240 mL	0.48	2.0
	Sports and Isotonic Drinks, Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	360 mL	0.36	1.0
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.08	0.8
	Toddler Formulas	100 mL[c]	0.06	0.6
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.04 to 0.85	5.0
	Other Drinks for Young Children	120 mL	0.07	0.6
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	0.8	20
	Cereal and Granola Bars	40 g	0.4	10
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk*	240 mL	0.24	1.0
Milk Products	Buttermilk*	240 mL	0.24	1.0
	Flavored Milk	240 mL	0.24	1.0
	Milk-Based Meal Replacement Beverages, for Weight Reduction[b]	240 mL	0.48	2.0
	Yogurt*	170 g	1.7	10

LNT = Lacto-*N*-tetraose; CFR = Code of Federal Regulations; RACC = Reference Amounts Customarily Consumed; U.S. = United States.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2018). When a range of values is reported for a proposed food-use, particular foods within that food-use may differ with respect to their RACC.

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

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

*LNT is intended for use in unstandardized products when standards of identity do not permit its addition.

From:	Christoph Röhrig
To:	Morissette, Rachel
Cc:	Marta Hanna Miks
Subject:	RE: questions for GRN 833
Date:	Wednesday, May 22, 2019 7:23:09 AM
Attachments:	image001.png image007.png Glycom Response to FDA Questions GRN 0008833 - May 22"19 - final.pdf GRAS Notice for lacto-N-tetraose - Revised page 5.pdf GRAS Notice for lacto-N-tetraose - Revised page 7.pdf GRAS Notice for lacto-N-tetraose - Revised page 13.pdf GRAS Notice for lacto-N-tetraose - Revised page 14.pdf GRAS Notice for lacto-N-tetraose - Revised pages 20-23.pdf

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Dear Rachel,

With many thanks for your kind patience do I provide the responses on FDA's letter from 3rd of May.

The last minute error that we noticed, and that caused the delay, is explained and addressed in the response on question 11.

Since your letter asked us not to provide an entire updated notice we do provide replacement pages specifically, when corrections were requested, or when errors were noticed.

In case that you would prefer an updated notice containing all corrections please let us know (some of the responses, e.g. question 12, weren't addressed by replacement pages since page breaks would have been introduced).

Please don't hesitate to get back to us in case of any questions you may find. I would kindly ask you to copy also my colleague Marta (here in cc).

Sincerely,
Christoph

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

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message and all copies and backups thereof immediately. Thank you.

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: 21. maj 2019 15:44
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: RE: questions for GRN 833

Hi Christoph,

Tomorrow will be fine.

Best,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

From: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Sent: Tuesday, May 21, 2019 9:31 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Subject: RE: questions for GRN 833

Dear Rachel,

While addressing FDA's questions on GRN833 we noticed a systematic error in some of our stability data tables literally last minute before sending you our response letter.

We have these currently fixed, and include the explanation and correction of the error in our response letter.

I trust we are able to send you the response letter now tomorrow. Apologies for the delay.

Kind regards,
Christoph

Christoph H. Röhrig
PhD, Senior Scientist

Head of Regulatory & Scientific Affairs

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: 6. maj 2019 15:35
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: RE: questions for GRN 833

Thank you!

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
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rachel.morissette@fda.hhs.gov

From: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Sent: Monday, May 06, 2019 9:28 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Subject: RE: questions for GRN 833

Dear Rachel,

OK, great, many thanks for these additional comments on the timing. The additional days may come in useful potentially.

I wish you safe travels for that weekend!

Kind regards,
Christoph

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

_____
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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: 6. maj 2019 15:19
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: RE: questions for GRN 833

Dear Christoph,

Thank you for your response. Yes, close of business on the 17th would be 10 business days, but don't worry if it's a few days late. I will be out of town until May 20, so if you send it by then that would be ok too. I won't see the responses until I check my email on the 21st anyway.

Best,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

FDA U.S. FOOD & DRUG ADMINISTRATION

From: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Sent: Monday, May 06, 2019 4:53 AM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Subject: RE: questions for GRN 833

Dear Dr. Morissette,

Thank you for sending us your questions on our GRAS notice for the human-identical milk oligosaccharide lacto-N-tetraose (LNT), filed by you as GRN 833.

I confirm receipt of your email, and trust that we will be able to respond to you within the requested 10 business days. Am I right to understand that means by the end of your office hours on Friday, May 17?

Kind regards,
Christoph

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

_____
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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: 3. maj 2019 17:51
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: questions for GRN 833

Dear Dr. Röhrig,

Please see attached questions to be addressed for GRN 000833. Please let me know if you have any questions at this time.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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Office of Food Additive Safety
U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

FDA U.S. FOOD & DRUG ADMINISTRATION

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

22 May 2019

Rachel Morissette
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740-3835 USA

Re: GRAS Notice No. GRN 000833

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s letter dated 03 May 2019 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for **lacto-N-tetraose (LNT)** filed by the Agency under GRN 833.

FDA.1. *On pages 4 and 5 of the notice, Glycom refers to "follow-on formula" and "toddler formula." As these terms do not have a regulatory definition, please describe what population is meant by these terms and whether they are the same or different.*

Follow-on formula and toddler formula are used interchangeably in the notice. Follow-on formula/toddler formula is defined by the World Health Organization as *"food intended for use as a liquid part of the weaning diet for the infant from the 6th month on and for young children*" (WHO, 2001[1]).

FDA.2. *Please clarify if LNT is intended to be formulated with other human milk-like oligosaccharides (HMOs), such as FOS, GOS, 2'-FL, LNnT, and other indigestible oligosaccharides on the market. What is the target level of total HMOs in infant formula?*

Similar to other human-identical milk oligosaccharides (HiMOs), LNT is intended for addition to infant formula to produce an infant formula that is compositionally representative of human breast milk. To this end, LNT may be added together with other human-identical milk oligosaccharides (HiMOs) such as LNnT and 2'-FL, to

[1] WHO (2001). *Follow-up Formula in the Context of the International Code of Marketing of Breast-Milk Substitutes*. (Briefing Note). World Health Organization (WHO). Available at: https://www.who.int/nutrition/follow-up_formula_eng.pdf.

match the levels in breast milk taking into account natural variation. Since Glycom is not manufacturing final infant nutrition products, we are not in the position to comment on the potential intention of infant nutrition manufacturers to combine HiMOs with other indigestible oligosaccharides on the market or on the target level of total HiMOs in infant formula.

Glycom is aware of the obligation of infant nutrition manufacturers to notify innovative infant formula recipes containing human-identical milk oligosaccharides − including LNT − with appropriate data before launching on the U.S. marketplace and trust that any given combination or addition level is supported specifically.

FDA.3. *Please clarify the food uses in infant and toddler foods. Does "other baby foods" (and corresponding RACC values) refer to all foods listed in 21 CFR 101.12, including cereal and grain products (ready-to-eat (RTE): 7 g infants, 20 g toddler; all others: 7 g); dinners, desserts, fruits, vegetables, soups (ready-to-serve (RTS) junior type or strained: 110 g; dry mix type: 15 g); RTS dinners, stews, or soups for toddlers: 170 g; RTS fruits for toddlers: 125 g; RTS vegetables for toddlers: 70 g; RTS egg/egg yolk: 55 g; and juice, all varieties: 120 mL?*

The proposed food-use "other baby foods" includes all foods targeted towards infants and young children 1 to 3 years of age. All food codes in the U.S. National Health and Nutrition Examination Survey (NHANES) 2013-2014 dataset described with the term "baby food" were assumed to target this population group and were selected for the intake assessment. Thus, the exposure assessment accounted for all foods for infants and young children listed in the 21 CFR §101.12, including cereal and grain products, dinners, desserts, fruits, vegetables, soups, ready-to-serve dinners, stews, soups, fruits, and vegetables. It should be noted that juices are included under the intended use of "other drinks for young children."

Furthermore, it should be noted that the Reference Amounts Customarily Consumed per Eating Occasion (RACCs) provided in Table 1.3-1 were for reference purposes only. Use levels applied in the intake assessment were those expressed on a g/kg or g/L basis and are thus dependent on the amount of food consumed rather than the serving size.

FDA.4. *Please revise the footnote in Table 1.3-1 "LNT is intended for use in unstandardized products when standards of identity do not permit its addition." to "LNT is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition."*

The footnote in Table 1.3-1 of the GRAS Notice has been revised to "*LNT is intended for use in unstandardized products and not in foods where standards of identity exist and do not permit its addition*." A replacement page (page 5) has been provided.

FDA.5. *Please correct the molecular formula for LNT shown on page 7 of the notice.*

We apologize for the typo on page 7 of the Notice, where the LNT molecular formula (on page 7) omitted the Nitrogen N ("$C_{26}H_{45}O_{21}$"). The correct molecular formula of LNT is $C_{26}H_{45}NO_{21}$. The correction has been implemented and a replacement page (page 7) has been provided.

FDA.6. *Please provide a description of the media and confirm if it is described in a prior GRN. Please also indicate if all media materials are food-grade. Please clarify what "allergen-free (except for milk-derived allergens)" means on page 12 of the notice. Is the media milk-based or does this refer to lactose?*

Previous references (e.g. GRN 650, GRN 659) to the growth media used for microbial production of similar human-identical milk oligosaccharides was limited to the general statement: "*Fermentation is performed in a chemically-defined, salt-based, minimal growth medium that excludes the use of antibiotics*."

The growth medium consists of the following raw materials and processing aids:

NAME	TECHNICAL FUNCTION	FORMULA
Purified water (PW)	Solvent	H_2O
Sucrose (saccharose) or glucose	Raw material	$C_{12}H_{22}O_{11}$ or $C_6H_{12}O_6$
Lactose monohydrate	Substrate	$C_{12}H_{22}O_{11} \times H_2O$
Ammonia gas	Nitrogen source	NH_3
Ammonium sulfate	Nitrogen source	$(NH_4)_2SO_4$
Phosphoric acid	Phosphor source	H_3PO_4
Potassium hydroxide	pH-adjustment	KOH
Citric acid hydrate	pH-adjustment	$C_6H_8O_7 \times H_2O$
Antifoam	Defoaming agent	
Magnesium sulphate heptahydrate	Essential element	$MgSO_4 \times 7\ H_2O$
Iron sulphate heptahydrate	Essential trace element	$FeSO_4 \times 7H_2O$
Manganese sulphate hydrate	Essential trace element	$MnSO_4 \times H_2O$
Zinc sulphate hydrate	Essential trace element	$ZnSO_4 \times H_2O$
Copper sulphate pentahydrate	Essential trace element	$CuSO_4 \times 5H_2O$
Nickel sulphate hexahydrate	Essential trace element	$NiSO_4 \times 6H_2O$
Sodium molybdate dihydrate	Essential trace element	$Na_2MoO_4 \times 2H2O$
Sodium selenite	Essential trace element	Na_2SeO_3
Thiamin HCl (Vitamin B_1)	Essential micronutrient (co-factor)	$C_{12}H_{17}N_4OS$

The following processing aids of non food-grade are used:

- Ammonia (gas)
- Trace metals: Nickel sulphate and sodium molybdate

Ammonia gas (NH_3) is used in the fermentation process as an acidity regulator and nitrogen source. Ammonia is a technical grade material and is supplied as a liquid (compressed gas). The relevant impurities according to the Joint FAO/WHO Expert Committee on Food Additives (JECFA) specification for 27 to 30% ammonium solution are non-volatile residues and lead (Pb). From the supplier we are informed that the content of non-volatile substances are below the limit set forward by JECFA. The limit of lead in the JECFA specification is 2 mg/kg for a 27 to 30% aqueous solution. The content of lead in the concentrated ammonia is typically less than 0.5 ppm. Lead is actively removed in downstream ion exchange process and the parameter is included in

the finished product specifications and monitoring plan. The added quantity of ammonia is not significant and from a worst-case point calculation the potential contamination from impurities stemming from ammonia, even if nothing would be removed (which is clearly not the case), will not exceed the limit in the finished product specification.

The transport of liquid ammonia is in dedicated tankers, so cross-contamination from transport vessels is not an issue.

Based on the content of potential impurities and the purification steps in the manufacturing process, it is evaluated that the use of technical grade ammonia is not of concern in the perspective of food safety and human health.

The **trace metals** nickel sulphate hexahydrate ($NiSO_4 \times 6H_2O$) and sodium molybdate dihydrate ($Na_2MoO_4 \times 2H_2O$) are added to the fermentation process and are processing aids serving the purpose to give the optimal conditions for growth of the production microorganism. Since the production microorganism is removed, and thus serves as a processing aid in the manufacture of the HMO product, the minerals could be considered to be "processing aids of a processing aid".

The trace metals are added in very small quantities (less than 10 ppm). Glycom wasn't able to source the trace metals as food grade, but they are of analytical grade and possess thus a very low content of impurities. The trace metals are actively removed in the downstream processing ion exchange step. Since these trace metals are not used as direct food additives and do not remain in the finished ingredient due to purification controls used during processing, the sources are not required to be food-grade certified.

Molybdate and Nickel were analysed in the finished product and the content is found to be below the possible detection limits.

It is evaluated that the addition of the trace metals, with the following downstream removal of metals in the ion exchange process, does not pose a risk to food safety and human health.

In regard to the terminology **"allergen-free (except for milk-derived allergens)"** we confirm that lactose is the only potential source of allergens due to possible presence of allergenic cow milk protein as trace impurities of the lactose raw material. All other raw materials and processing aids are sourced from the suppliers including major allergen declarations in accordance with the Food Allergen Labeling and Consumer Protection Act of 2004.

FDA.7. *On page 11 of the notice, there is a statement that "All additives, processing-aids, and food contact articles used during manufacturing are permitted by federal regulation, have been previously determined to be GRAS for their respective uses, or have been the subject of an effective food contact notification." A summary table is provided indicating the processing steps, but removal of impurities is not detailed. If the details of the manufacturing process are the same or similar to those in GRN 000659 (LNnT), please state that. If the data and information from GRN 000659 is being incorporated into the notice, please provide a statement saying so. Otherwise, please provide details on the removal of impurities.*

Principally, the down-stream processing (DSP) operations of the LNT manufacturing process are largely similar to the ones described in GRN 000659 (LNnT), with one major difference, that the LNT ingredient is not subjected to a crystallization step at the end of the DSP purification procedure. Consequently, the remaining purification steps of the LNT DSP have been individually optimized to reduce the number and amount of

impurities and product-related carbohydrates and to provide a high-purity material (without the need for a crystallization step). The production changes also eliminate the use of organic solvents in the production of LNT (when compared to LNnT) since the organic solvents were exclusively required for the crystallization step. The schematic overview of the manufacturing process for LNT with a more detailed description on the removal of impurities is presented in Table 1 below.

Table 1 Overview of the Manufacturing Process for the LNT Product

STAGE 1		**Upstream Processing (USP)**	**Description**
STEPS	**1**	Media Preparation	
	2	Propagation - Working cell bank (WCB)	
	3	Seed Fermentation	
	4	Fermentation Phases:	Production of LNT
	4A	Growth (Batch) Phase	
	4B	Feeding (Fed-Batch) Phase	
	5	Removal of Microorganism* - Ultrafiltration/diafiltration (UF/DF)	Removal of cells and large biomolecules (*e.g.*, protein, nucleic acids and lipopolysaccharides)
STAGE 2		**Downstream Processing (DSP)**	
STEPS	**6**	Purification/Concentration 1* - Nanofiltration or nanofiltration/diafiltration (NF1/DF) membranes	Reduction water, minerals and very small biomolecules
	7	Ion Removal - Ion exchange resin (IEX)	Removal of charged molecules and salts (*e.g.*, trace metals)
	8	Decolorization* - Active charcoal (AC)	Removal of colored and predominantly lipophilic impurities by active charcoal adsorbent
	9	Purification/Concentration 2* - Nanofiltration or nanofiltration/diafiltration (NF2/DF) membranes	Reduction water, minerals and very small biomolecules
	10	Drying - Spray/freeze drying	Removal of water
	11	Sampling and Packaging	
	12	Quality Control and Batch Release	Specifications are tested, and CoA issued

LNT = lacto-*N*-tetraose.

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

FDA.8. *Please describe how any microbial endotoxins are efficiently removed by the production process.*

Gram-negative bacterial endotoxins (also called pyrogens or lipopolysaccharides) are amphiphilic, structurally complex, high molecular weight glycolipid macromolecules, which consist of a hydrophobic lipid core-structure (lipid A) and a hydrophilic, charged and complex oligo- to polysaccharide structure component. The structure of lipid A contains a number of fatty acid esters, rendering it lipophilic, but is typically also phosphorylated adding further charges to the complex molecule. An illustrative example of a schematic overview on the complex chemical structure of *E. coli* endotoxin has been reproduced from Magalhães *et al.* (2007)[2] in Figure 1 below (Magalhães *et al*., 2007).

The fundamentally different physico-chemical properties of endotoxins − as compared to hydrophilic, small molecular weight human-identical milk oligosaccharides like LNT − make purification and removal of

[2] Magalhaes, P. O., Lopes, A. M., Mazzola, P. G., Rangel-Yagui, C., Penna, T. C. & Pessoa, A., Jr. **2007.** Methods of endotoxin removal from biological preparations: a review. *J. Pharm. Pharm. Sci.,* 10**,** 388-404.

endotoxins comparatively straightforward. Endotoxins are effectively reduced during the ultrafiltration step (due to their large molecular weight), the cation and anion removal steps (due to their electric charges) and the active charcoal processing step (due to their lipophilic structure components).

To ensure the efficient removal of endotoxins Glycom has introduced a specification for endotoxin at 10 Endotoxin Unit per gram for all of its human-identical milk oligosaccharides. The risk assessment of these specified maximum levels has been previously discussed as response to FDA questions on GRN 000650.

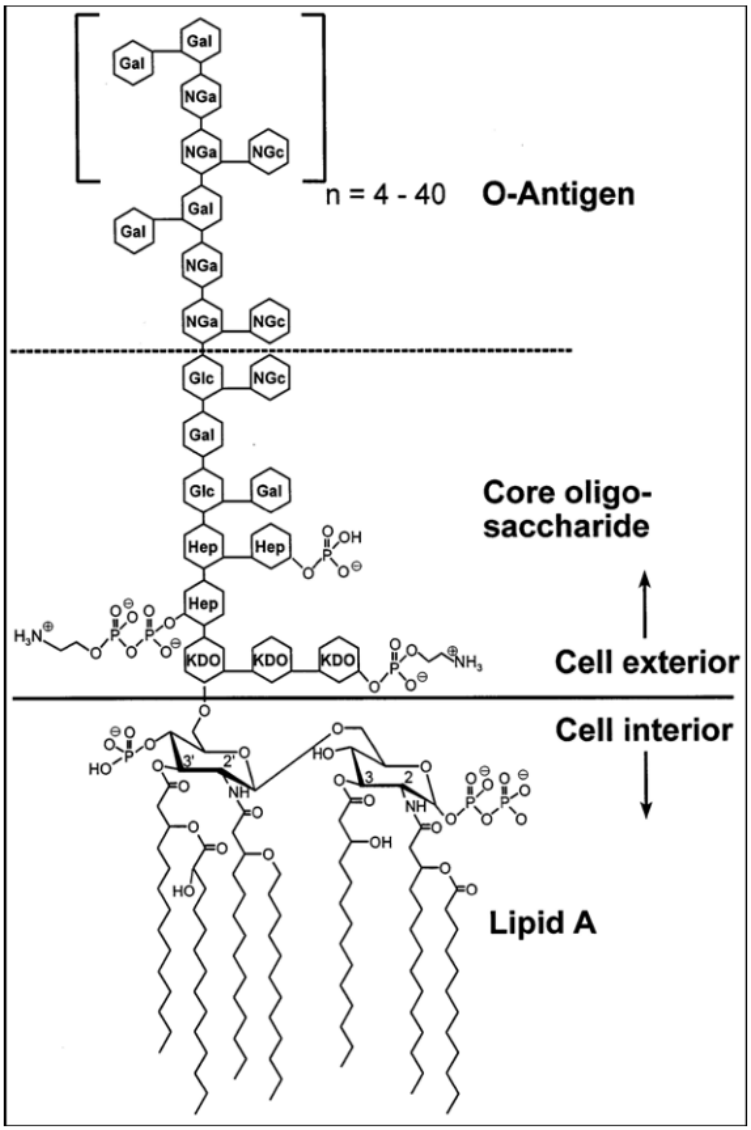

Figure 1. Chemical structure of endotoxin from *E. coli* O111:B4 according to Ohno and Morrison (1989)[3].

(Hep) L-glycerol-D-manno-heptose; (Gal) galactose; (Glc) glucose; (KDO) 2-keto-3-deoxyoctonic acid; (NGa) N-acetyl-galactosamine; (NGc) N-acetyl-glucosamine.

[3] Ohno, N. & Morrison, D. C. **1989.** Lipopolysaccharide interaction with lysozyme. Binding of lipopolysaccharide to lysozyme and inhibition of lysozyme enzymatic activity. *J. Biol. Chem.,* 264**,** 4434-41.

FDA.9. *Please provide a specification limiting lead and three non-consecutive batch analyses to show LNT can be manufactured to meet that specification.*

Glycom has included a specification for lead of 0.1 mg/kg as monitored by the internationally recognized methods EN 13805:2002 and EPA-6020A:2007. Table 2.3.1-1 of the GRAS Notice has been revised with the lead specification. Glycom notes that the GRAS Notice already included batch data for lead (see Table 2.3.3.4-1). A replacement page (page 14) has been provided.

We also noticed another error in the specification table 2.3.1-1, which referred to an analytical method by the wrong name: method HPLC-703-7C7-006 should read HPAEC-HMO-006. This error occurred in the lines of Assay (water free) HiMS, D-lactose, lacto-N-triose II, para-LNH2 and sum of other carbohydrates. These errors have been corrected in replacement pages 13 and 14. We apologize for the error.

FDA.10. *Please list the "other carbohydrates" limited to NMT 5.0% by specification.*

The "other carbohydrates" are comprised of lactulose, lactitol, glucose, galactose, fructose, ribose, the reduced form of LNT ("Gal-GlcNAc-Gal-Sorbitol"), isomaltose, lacto-*N*-triose II fructose isomer ("GlcNAc-Gal-fructose"), GlcNAc-LNT and 3-Gal-LNT. The main contribution derives from the reduced carbohydrates (lactitol and reduced form of LNT) and isomaltose.

FDA.11. *Please comment on the ingredient that was used in the 5-year stability study on page 20 of the notice. It does not meet the assay (water free) HiMS minimum of 90%. Does this reflect a change in the processing method?*

Upon investigating the request, we noticed two errors in **Tables 2.4.1.1-1** (real-time stability data), **A** (batch CPN4215 00115) and **B** (batch CPN4215 1000516 FD):

(1) the data presented as real-time stability data has mistakenly been exchanged with the data from the accelerated stability studies. This has been a copy-paste error for which we apologize. The real-time stability data did not include the time points at 1 and 2 months.

(2) a miscalculation of the parameter "Assay (water free) HiMS", which erroneously did not correct for the water content. To calculate this parameter in its "water free" form, it is required to add the sum of human-identical milk saccharides (i.e. LNT, lactose and lacto-N-triose II) and to correct the assay value then for the water content. The correction for water was accidentally forgotten. As comment we want to add that assay values are not corrected for the water content, unless explicitly noted.

The first error was corrected by providing now replacement pages (20 and 21) that contain the correct data for the real-time stability studies. The second error was also corrected in the updated tables of the replacement pages.

The same miscalculation of the "Assay (water free) HiMS" parameter also occurred in **Tables 2.4.1.2-1 A** and **B**, and is now corrected by providing two additional corrected replacement pages (22 and 23).

Since filing of the notice further time-points of the real-time stability studies have become available which we added in Tables 1 A and B below.

Table 1 Results of the 5-Year Real-Time Stability Study on LNT product (25°C, 60 % Relative Humidity, RH) for 2 representative batches A) No CPN4215 00115

A) Manufacturing Batch Number CPN4215 00115

Parameter	Sample Time (Months)							
	0	3	6	9	12	18	24	36
Physical Properties								
Color	White	White	White	White	Ivory white	White	Ivory white	Ivory white
Appearance	Non-agglutinated powder	Agglutinated powder	Agglutinated powder	Agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder	Agglutinated powder
Purity								
Water content [%]	13.6	11.9	12.2	12.4	12.5	15.6	13.0	14.0
Assay – LNT [%]	78.3	79.8	79.6	77.9	77.3	78.2	76.3	78.8
Lactose [%]	1.74	1.89	1.86	1.91	1.94	1.26	1.2	1.9
Lactulose [%]	n.r.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Lacto-N-triose II [%]	4.0	4.33	4.11	4.68	4.79	3.25	3.4	3.9
Para-LNH2 [%]	0.68	0.66	0.67	0.65	0.66	0.78	0.8	0.8
LNT fructose isomer [%]	0.38	0.35	0.31	0.39	0.36	0.33	0.4	0.3
Assay (water free) HiMS [%]	97.3	97.6	97.5	96.4	96.0	97.9	93.0	98.4
Microbiological Quality								
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	< 10
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Absent in 10 g
Salmonella spp.	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Not tested	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Absent in 10 g
Listeria monocytogenes	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Not tested	Absent in 25 g
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	< 10
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	< 10
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	< 10

CFU = Colony Forming Unit, LNT = Lacto-*N*-tetraose, para-LNH2 = para-Lacto-N-hexaose-2, HiMS = Human-identical Milk Saccharides = Sum of LNT, lactose and lacto-*N*-triose II, n.r. = not reported, n.a. = not available.

B) Manufacturing Batch Number CPN4215 1000516 FD

Parameter	Sample Time (Months)						
	0	3	6	9	12	18	24
Physical Properties							
Color	White	n.a.	n.a.	n.a.	White	White	Not tested
Appearance	Fine powder	n.a.	n.a.	n.a.	Agglutinated powder	Powder with agglomerates	Not tested
Purity							
Water content [%]	2.4	2.2	1.8	2.2	2.3	3.0	Not tested
Assay – LNT [%]	74.9	76.3	76.9	73.4	75.6	75.0	Not tested
Lactose [%]	8.7	8.9	9.30	8.8	8.6	9.3	Not tested
Lactulose [%]	0.2	0.2	0.2	0.20	0.2	0.2	Not tested
Lacto-N-triose II [%]	5.7	5.5	5.6	5.6	5.6	6.0	Not tested
Para-LNH2 [%]	2.1	2.0	2.3	2.2	2.0	2.1	Not tested
LNT fructose isomer [%]	1.0	0.9	0.9	1.0	0.9	1.0	Not tested
Assay (water free) HiMS [%]	91.5	92.7	93.5	89.7	91.9	93.1	NA
Microbiological Quality							
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested
Salmonella spp.	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Not tested
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested
Listeria monocytogenes	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Not tested
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested

CFU = Colony Forming Unit, LNT = Lacto-*N*-tetraose, para-LNH2 = para-Lacto-N-hexaose-2, HiMS = Human-identical Milk Saccharides = Sum of LNT, lactose and lacto-*N*-triose II, n.r. = not reported, n.a. = not available.

Furthermore, additional data has become available for the accelerated stability trial of batch CPN4215 1000516 FD which we have added to Table 2 B below.

Since these additional data points were not available at the time of the initial filing of the notice, we have not included these data in the replacement pages that we provided for the other errors identified by FDA and us. However, if FDA prefers we are happy to provide an updated GRAS notice incorporating all of the corrections and these updates upon request.

Table 2 Representative Interim Results of the 2-Year Accelerated Stability Study on LNT product (40°C, 75% Relative Humidity, RH) for representative batch B) CPN4215 1000516 FD.

B) Manufacturing Batch Number CPN4215 1000516 FD

Parameter	Sample Time (Months)							
	0	1	2	3	6	9	12	18
Physical Properties								
Color	White	White	White	White	White	White	White	White
Appearance	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Agglutinated powder	Powder with agglomerates
Purity								
Water content [%]	2.4	2.3	2.3	2.2	1.8	2.0	2.3	3.0
Assay – LNT [%]	74.9	75.0	75.7	77.7	76.9	74.4	75.9	74.9
Lactose [%]	8.7	8.6	9.1	9.2	9.0	8.2	8.8	9.2
Lactulose [%]	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Lacto-*N*-triose II [%]	5.7	5.9	5.8	5.6	5.6	5.6	5.7	5.9
Para-LNH2 [%]	n.a.	2.0	2.0	2.0	2.2	2.0	2.1	2.1
LNT fructose isomer [%]	1.0	1.0	0.8	1.0	0.9	1.0	0.9	1.1
Assay (water free) HiMS [%]	91.5	91.6	92.8	94.6	93.2	90.0	92.6	92.7
Microbiological Quality								
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested
Salmonella spp.	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested
Listeria monocytogenes	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested

CFU = Colony Forming Unit, LNT = Lacto-*N*-tetraose, para-LNH2 = para-Lacto-N-hexaose-2, HiMS = Human-identical Milk Saccharides = Sum of LNT, lactose and lacto-*N*-triose II, n.r. = not reported, n.a. = not available.

FDA.12. *The exposure estimates provided in the notice do not include infants ages 0-12 months. Please provide an estimate of intake for LNT and total added HMOs for infants that consume infant formula only (up to 4-6 months of age) and older infants (approximately 6-12 months of age) that consume both infant formula and infant foods. If an estimate from a previous GRN is used, please incorporate this data and information into the notice with a brief description and provide the appropriate page numbers where this information is found in the prior notice.*

The intake estimates for LNT among infants (aged 0 to 6 months and 7 to 12 months) are provided below in Tables 2 and 3 on an absolute and body weight basis, respectively. As noted in response to question #2 above, LNT and other HiMOs are added to infant formula to produce a formula that is compositionally representative of human breast milk, taking into account the natural variation of HMOs.

On an absolute basis, the mean and 90th percentile consumer-only intakes of LNT among infants aged 0 to 6 months were determined to be 1.21 and 2.20 g/person/day, respectively (Table 2). Among infants aged 7 to 12 months, the mean and 90th percentile consumer-only intakes of LNT were determined to be 1.75 and 3.28 g/person/day, respectively. Glycom notes that these exposures fall within the upper range of expected intakes of LNT from infant consumers of human milk where concentrations of up to 3.8 g/L have been reported (see Section 3.1.3 of GRN 000833).

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

Population Group	Age Group (Months)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Young Infants	0 to 6	0.97	2.04	80.1	165	1.21	2.20
Older Infants	7 to 12	1.75	3.28	99.9	127	1.75	3.28

LNT = lacto-N-tetraose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.
[a] Intake data expressed as wet weight of ingredient under the proposed conditions of intended use.

On a body weight basis, the mean and 90th percentile consumer-only intakes of LNT among infants aged 0 to 6 months were determined to be 176 and 301 mg/kg body weight/day, respectively (Table 3). Among infants aged 7 to 12 months, the mean and 90th percentile consumer-only intakes of LNT were determined to be 197 and 352 mg/kg body weight/day, respectively.

Table 3 Summary of the Estimated Daily Per Kilogram Body Weight Intake of LNT[a] from Proposed Food-Uses in the U.S. by Infants (2013-2014 NHANES Data)

Population Group	Age Group (Months)	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Young Infants	0 to 6	141	286	80.1	165	176	301
Older Infants	7 to 12	197	352	99.9	127	197	352

bw = body weight; LNT = lacto-N-tetraose; 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.

These missing data has not been provided via replacement pages to the notice since page breaks would be inserted changing the paging of the remaining notice. Glycom would be happy to provide an entire updated notice upon request but noted that this was not wished for in the questions by FDA.

FDA.13. *Please clarify if the exposure estimate provided for toddlers 1-3 years of age includes consumption of toddler formula.*

Exposure estimates provided on both an absolute and body weight basis for toddlers aged 1 to 3 years of age includes the consumption of toddler formula.

FDA.14. *Levels of most constituents in human milk change during the lactation period. However, Glycom proposes a single use level (0.8 g/L) in infant formula for ages 12 months and younger. Glycom states on page 27 of the notice that the levels of LNT decline in samples obtained from lactation stages >2 months. In Table 3.1.2.2-1, the average levels of LNT for Days 1-4, Days 5-14, Days 10-60, and >2 months are listed as 0.8, 0.9, 1.1, and 0.5 g/L, respectively. Thus, infants >2 months presumably would have a higher exposure to LNT in infant formula containing 0.8g/L than infants who are naturally exposed to LNT through breastmilk. Given the implicit intention of making infant formula more like human milk, please provide a rationale why 0.8g/L of LNT is appropriate for infants >2 months old.*

Glycom is proposing a single maximum level of 0.8 g/L for the addition of LNT to infant formulas below 12 months of age to allow for larger flexibility for the manufacturers of infant nutrition. The basis of safety is established by (1) an exposure estimate on kilogram body weight basis and by (2) the large reported ranges that are known to occur in human breastmilk for human milk oligosaccharides in general and LNT in particular. Published data beyond 2 months of lactation has been comparably scarce for LNT until recently but Coppa *et al.* (1999) reported LNT to occur at concentrations around 1.3 g/L in secretor milk samples from day 90 of lactation (Coppa *et al.,* 1999[4]). Data published more recently – which was not yet included in Glycom's GRAS Notice for LNT – report LNT levels in Canadian human milk samples from 3-4 months of lactation between 0.94 and 1.32 g/L (Azad *et al.,* 2018[5]) and in Malaysian human milk samples from 1 year of lactation at 1.16 g/L (Ma *et al*. 2018[6]).

Glycom is aware of the obligation of infant nutrition manufacturers to notify innovative infant formula recipes containing human-identical milk oligosaccharides − including LNT − with appropriate data before launching on the U.S. marketplace and trust that any given addition level is supported specifically.

FDA.15. *It is unclear in the notice whether LNT will be used in conjunction with other HMOs. If the intended use involves co-addition of other HMO-like substances, please indicate how the total load of indigestible oligosaccharides will be kept below levels of concern (i.e. causing adverse gastrointestinal effects).*

Glycom is a manufacturer of infant formula ingredients, not a manufacturer of infant formula; therefore, Glycom is not in a position to comment on the appropriateness of using LNT with other HMO-like substances. Glycom notes that all infant formulas marketed in the U.S. must meet federal nutrient requirements and infant

[4] Coppa, G. V., Pierani, P., Zampini, L., Carloni, I., Carlucci, A. & Gabrielli, O. **1999.** Oligosaccharides in human milk during different phases of lactation. *Acta Paediatr. Suppl.,* 88**,** 89-94.

[5] Azad, M. B., Robertson, B., Atakora, F., Becker, A. B., Subbarao, P., Moraes, T. J., Mandhane, P. J., Turvey, S. E., Lefebvre, D. L., Sears, M. R. & Bode, L. **2018.** Human Milk Oligosaccharide Concentrations Are Associated with Multiple Fixed and Modifiable Maternal Characteristics, Environmental Factors, and Feeding Practices. *J. Nutr.,* 148**,** 1733-1742.

[6] Ma, L., McJarrow, P., Jan Mohamed, H. J. B., Liu, X., Welman, A. & Fong, B. Y. **2018.** Lactational changes in the human milk oligosaccharide concentration in Chinese and Malaysian mothers' milk. *Int. Dairy J.,* 87**,** 1-10.

formula manufacturers must notify the FDA prior to marketing a new formula. Under Section 412(d)(1) of the Federal Food, Drug, and Cosmetic Act (FFDCA), a manufacture of a new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula, and this must include, among other things, a description of any reformulation of the formula or change in processing of the infant formula. Accordingly, the manufacturer will need to provide the Agency with information supporting that a particular oligosaccharide Combination (*e.g*., use of LNT with an indigestible oligosaccharide such as GOS) would be well tolerated as part of the Agency's 90-day notification procedure. It is therefore Glycom's view that existing regulations governing the pre-market clearance requirements for infant formula in the U.S. are sufficient to ensure that a particular combination of indigestible oligosaccharides that may be used in a new infant formula product are safe and suitable for their intended use.

FDA.16. *According to Sprenger et al. (2017), who examined if there is a dependence of certain HMOs on FUT2 status (using 2'-FL as a marker), the levels of LNT and LNnT are correlated with 2'-FL levels negatively and positively, respectively. If the intended use involves LNT in the presence of 2'-FL (as in LNnT uses), please state if the levels of LNT will be adjusted. If not, please discuss why there is no safety concern.*

It is correct that Sprenger *et al.* (2017)[7] − and also Azad *et al.* (2018)[8], Kunz *et al.* (2017)[9], McGuire *et al.* (2017)[10], Hong *et al.* (2014)[11], and Thurl *et al*. (2010)[12] − report higher LNT levels in the milk of non-secretor mothers (who do not express 2'-FL into their milk) than in the milk of secretor mothers (whose milk contains 2'-FL). On average − on basis of these published data − the levels of LNT in non-secretor mothers was around 1.6 to 1.7 g/L, whereas around 1.0 g/L in the milk of secretor mothers. The proposed use levels at a maximum of 0.8 g/L are thus already on the conservative end of the reported spectrum.

Glycom is not in the position at this stage to comment on the intended recipes of infant formula manufacturers about relative concentrations of LNT and other human-identical milk oligosaccharides like 2'-FL.

Glycom is aware of the obligation of infant nutrition manufacturers to notify innovative infant formula recipes containing human-identical milk oligosaccharides − including LNT − with appropriate data before launching on the U.S. marketplace and trust that any given addition level is supported specifically.

[7] Sprenger, N., Lee, L. Y., De Castro, C. A., Steenhout, P. & Thakkar, S. K. **2017.** Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study. *Plos One,* 12**,** e0171814. DOI 10.1371/journal.pone.0171814.

[8] Azad, M. B., Robertson, B., Atakora, F., Becker, A. B., Subbarao, P., Moraes, T. J., Mandhane, P. J., Turvey, S. E., Lefebvre, D. L., Sears, M. R. & Bode, L. **2018.** Human Milk Oligosaccharide Concentrations Are Associated with Multiple Fixed and Modifiable Maternal Characteristics, Environmental Factors, and Feeding Practices. *J. Nutr.,* 148**,** 1733-1742.

[9] Kunz, C., Meyer, C., Collado, M. C., Geiger, L., Garcia-Mantrana, I., Bertua-Rios, B., Martinez-Costa, C., Borsch, C. & Rudloff, S. **2017.** Influence of Gestational Age, Secretor and Lewis Blood Group Status on the Oligosaccharide Content of Human Milk. *J. Pediatr. Gastroenterol. Nutr.,* 64**,** 789-798.

[10] McGuire, M. K., Meehan, C. L., McGuire, M. A., Williams, J. E., Foster, J.*, et al.* **2017.** What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. *Am. J. Clin. Nutr.,* 105**,** 1086-1100.

[11] Hong, Q., Ruhaak, L. R., Totten, S. M., Smilowitz, J. T., German, J. B. & Lebrilla, C. B. **2014.** Label-free absolute quantitation of oligosaccharides using multiple reaction monitoring. *Anal. Chem.,* 86**,** 2640-7.

[12] Thurl, S., Munzert, M., Henker, J., Boehm, G., Mueller-Werner, B., Jelinek, J. & Stahl, B. **2010.** Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br. J. Nutr.,* 104**,** 1261-1271.

FDA.17. *On page 33 of the notice, Glycom states:*

"As mentioned previously, LNnT is a chemically and structurally similar isomer

of LNT. Therefore, the toxicological data obtained for LNnT preparations are considered relevant in supporting the safety of LNT."

However, several publications not listed in Part 7 indicate that the two isomers may have different bioactivities (Bohari et al., 2016; Ozcan and Sela, 2018). In fact, Ozcan and Sela conclude:

"… differential metabolism of milk glycans potentially drives the emergent physiology of host-microbial interactions to impact infant health."

While it is unlikely, given the structural similarities, that LNT and LNnT would have significantly different toxicological profiles, the potentially differential effects on infants' gut microbiota by LNT and LNnT may impact tolerability. Please discuss any differences between LNT and LNnT and why these differences do not impact safety.

Glycom acknowledges subtle differences in the biological function of LNnT and LNT, which are at least in part a result of differentiated metabolism by human gut microbiota, predominantly commensals like bifidobacteria, and in part related to different receptor recognition (either by receptors on human gut cells, symbiotic bacteria or pathogens) of the different terminal linkage being either a ß(1-4) or ß(1-3)-galactose bond, which results in a different three-dimensional structural space of the two isomeric molecules. Indeed, the differentiated metabolism *via* different specific enzymes has been elucidated and reported for a couple of bifidobacteria species and strains (*e.g.*, Kitaoka, 2012[13]; Yoshida *et al.* 2012[14]). However, both human milk oligosaccharides have clearly been reported to be both bifidogenic, independent of the distinct utilization mechanism (*e.g.*, Asakuma *et al.* 2011[15];).

The subtle structural differences could theoretically produce slightly different tolerability profiles when considering the safety of ingesting supraphysiological levels of LNnT and LNT that are outside the ranges reported for human milk. However, in Glycom's opinion, the proposed use level of LNT is below any expected tolerability limitation since it is clearly within the natural levels of LNT in human milk.

The statement made in the notice in respect to the relevance of the LNnT animal toxicology data for the safety of LNT does not pertain to tolerability effects, but clearly to toxicological safety. The toxicological safety of HiMO preparations are hardly a matter of the identity of the products, which are indeed structure-identical to their corresponding counterparts in breastmilk, and are thus inherently safe under the condition that natural exposure levels are not exceeded. Rather, it is the potential impurity profile caused by the nature of the manufacturing process that is investigated by overdosing in animal toxicology studies. In that sense, Glycom believes that the toxicology data on LNnT is indeed of relevance for the safety profile of LNT as well, since both manufacturing processes are largely similar.

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[13] Kitaoka, M. **2012.** Bifidobacterial Enzymes Involved in the Metabolism of Human Milk Oligosaccharides. *Adv. Nutr.,* 3**,** 422S-429S.

[14] Yoshida, E., Sakurama, H., Kiyohara, M., Nakajima, M., Kitaoka, M., Ashida, H., Hirose, J., Katayama, T., Yamamoto, K. & Kumagai, H. **2012.** Bifidobacterium longum subsp. infantis uses two different β-galactosidases for selectively degrading type-1 and type-2 human milk oligosaccharides. *Glycobiology,* 22**,** 361-368.

[15] Asakuma, S., Hatakeyama, E., Urashima, T., Yoshida, E., Katayama, T., Yamamoto, K., Kumagai, H., Ashida, H., Hirose, J. & Kitaoka, M. **2011.** Physiology of the consumption of human milk oligosaccharides by infant-gut associated bifidobacteria. *J. Biol. Chem.,* 286**,** 34583-34592.

FDA.18. *On page 40 of the notice, Glycom makes the following statement in support of its argument that EFSA's choice of NOAEL for LNnT should be higher than 2500 mg/kg bw/day:*

"... the apparent changes reported by the NDA Panel [EFSA] have not been reported in subsequent subchronic toxicity evaluations of LNnT (or LNT) conducted in neonatal rats at doses up to 5000 mg/kg body weight/day (Penard, 2016; Phipps et al., 2018)."

Given that the Phipps et al. (2018) study on LNT was dosed up to 4000 mg/kg bw/day, please revise this statement.

Glycom notes that in contrast to the study on LNnT in which the highest tested dose was 5,000 mg/kg body weight/day, the dose of LNT used in the 90-day study in neonatal rats was limited to 4,000 mg/kg body weight/day due to the solubility of the substance (higher concentrations were too viscous to be dosed to the animals by oral gavage). The LNnT batch used in the 90-day study contained approximately 99% LNnT, whereas the LNT batch used in the 90-day study contained only 77% LNT, the remainder composed of other carbohydrates such as lactose, lacto-*N*-triose II, and *para*-lacto-N-hexaose-2. Thus, doses in the LNT study were adjusted for LNT content and the 4,000 mg/kg body weight/day LNT dose is equivalent to approximately 5,200 mg/kg body weight/day of total oligosaccharides. Nevertheless, the GRAS Notice has been revised with the following statement to clearly differentiate the doses of LNnT and LNT:

"the apparent changes identified by the NDA Panel have not been reported in a subsequent 90-day toxicity study of LNnT in neonatal rats provided gavage doses up to 5,000 mg/kg body weight/day, or in a subchronic feeding study of LNT at levels providing 4,000 g/kg body weight per day (Penard, 2016; Phipps et al., 2018)."

FDA.19. *On page 5 of the GRAS Panel summary, the Phillips et al. (2018) reference is incorrect and refers to a different paper. Please provide the correct reference and confirm that the GRAS Panel reviewed the correct reference.*

Glycom notes that the GRAS Panel reviewed the correct publication for LNT and the citation was incorrect.

We hope this information adequately addresses the Agency's questions on GRN 000833, and if there is any additional information or further clarification that is required, Glycom will happy to provide such information upon request.

Sincerely,

22 May 2019

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

Date

beverages targeted towards the general U.S. population (up to 1.0 g/L or 10 g/kg), and foods for special dietary use (*e.g.*, meal replacement bars) at levels up to 2.0 g/L or 20 g/kg. The maximum use-levels are proposed on the basis of providing similar levels of LNT on a body weight basis as those consumed by breast-fed infants (see Section 3.1.3).

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

Food Category	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use-Level (g/RACC)	Proposed Maximum Use-Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction[b]	240 mL	0.48	2.0
	Sports and Isotonic Drinks, Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	360 mL	0.36	1.0
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.08	0.8
	Toddler Formulas	100 mL[c]	0.06	0.6
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.04 to 0.85	5.0
	Other Drinks for Young Children	120 mL	0.07	0.6
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	0.8	20
	Cereal and Granola Bars	40 g	0.4	10
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk*	240 mL	0.24	1.0
Milk Products	Buttermilk*	240 mL	0.24	1.0
	Flavored Milk	240 mL	0.24	1.0
	Milk-Based Meal Replacement Beverages, for Weight Reduction[b]	240 mL	0.48	2.0
	Yogurt*	170 g	1.7	10

LNT = Lacto-*N*-tetraose; CFR = Code of Federal Regulations; RACC = Reference Amounts Customarily Consumed; U.S. = United States.

[a] RACC based on values established in 21 CFR §101.12 (U.S. FDA, 2018aa). 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.

* LNT 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

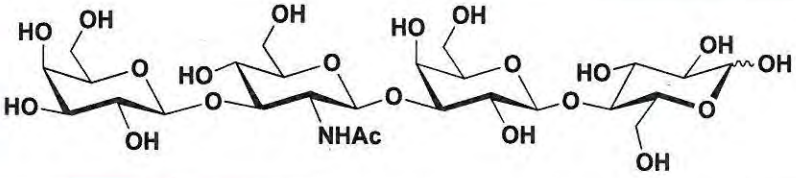
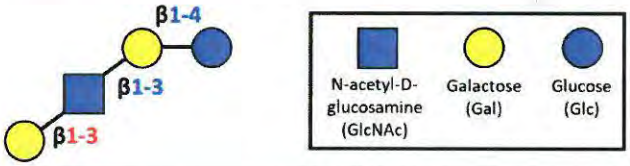
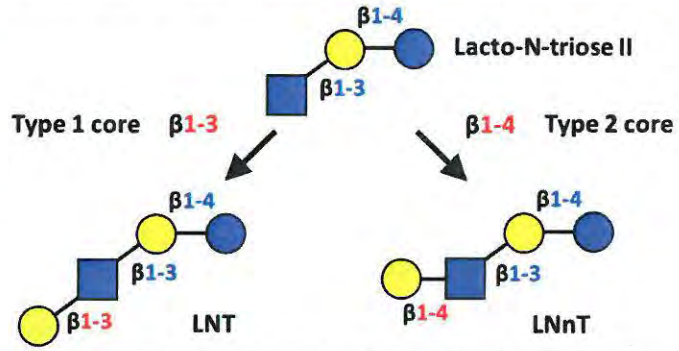
Pursuant to 21 CFR § 170.30 (a)(b) of the Code of Federal Regulations (CFR) (U.S. FDA, 2018b), Glycom has concluded that the intended uses of LNT as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

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

Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

Table 2.1-1 Description of Identity of LNT

Product Name	Lacto-*N*-tetraose
Abbreviations	LNT
IUPAC Name	β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
IUPAC Abbreviation (extended)	β-D-Gal*p*-(1-3)-β-D-GlcNAc*p*-(1-3)-β-D-Galp-(1-4)-D-Glc
IUPAC Abbreviation (condensed)	Gal-(β1-3)-GlcNAc-(β1-3)-Gal-(β1-4)-Glc
Molecular Structure	
Symbol Nomenclature	
Molecular Formula	$C_{26}H_{45}NO_{21}$
Molecular Mass (weight)	707.63
CAS Number	14116-68-8
CAS Name	D-Glucose, O-β-D-galactopyranosyl-(1→3)-O-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-
The structural relationship between LNT, LNnT and lacto-*N*-triose II	
	

CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry; LNnT = Lacto-*N*-neotetraose; LNT = Lacto-*N*-tetraose.

2.2 Manufacturing

2.2.1 Description of the Production Microorganism

2.2.1.1 Parental (Host) Strain

The genotypic characteristics of the parental/recipient microorganism, *Escherichia coli* K-12 DH1, are presented in Table 2.2.1.1-1. The genome of *E. coli* K-12 has been sequenced and bioinformatic

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

Specifications for LNT are presented in Table 2.3.1-1. The parameters include the main components of the HiMS mixture, consisting predominantly of LNT (min. 70%), with some levels of lactose (max. 12%) and lacto-*N*-triose II (max. 10%), as well as possible by-products and degradation products[9] such as *para*-lacto-*N*-hexaose-2 (max. 3.5%) and the LNT fructose isomer[10] (max. 1%).

LNT is further specified as a white to off-white powder that is produced by a microbial fermentation process using an *E. coli* K-12 DH1-derived strain. Since lactose and lacto-*N*-triose II are also naturally present in human milk, another quality parameter relevant to the infant nutrition uses has been introduced with 90.0% as the sum of HiMS to ensure a highly consistent product quality in context of that particular proposed use. The LNT content is specified by water-free assay based on high-performance anion exchange chromatography (HPAEC) coupled with pulsed amperometric detection (PAD) analysis. The main advantages of the PAD detector used is high sensitivity and selectivity, wide dynamic detection levels, reliable performance with excellent precision and more consistent response over wide range of chemical structures (*e.g.*, no chromophores are required for detection). Upper limits have also been established for microbiological parameters and includes separate specifications for LNT that is used during the wet blending stage of infant formula where heat killing steps are applied prior to dry-blending of heat-sensitive ingredients; infant formula containing LNT will therefore be compliant with the microbial requirements for infant formula as defined under 21 CFR §106.55. These microbial specifications are comparable to limits concluded to be GRAS for the addition of galacto-oligosaccharides to infant formula as described in GRN 620.

All methods of analysis are either internationally-recognised or developed and validated internally by Glycom and confirmed by independent accredited external laboratories [International Laboratory Accreditation Cooperation (ILAC)-accredited laboratory WESSLING Hungary Kft., and Eurofins Medigenomix GmbH (Germany) accredited against ISO/IEC 17025:2005 by Die Deutsche Akkreditierungsstelle GmbH (DakkS)].

Table 2.3.1-1 Specifications for LNT

Definition				
Lacto-*N*-tetraose (LNT) is a purified, white to off-white powder that is produced by a microbial process.				
A modified strain of *Escherichia coli* K-12 DH1.				
Parameter	**Specification**	**AVE**	**± SD**	**Method**
Appearance	Powder or agglomerates	Complies		ISO 6658:2007
Colour	White to off white	Complies		ISO 6658:2007
Identification (LNT)	RT of standard ± 3%	Complies		Glycom method HPLC-703-7C7-001
Assay (water free) HiMS[a]	Not less than 90.0 w/w %	93	± 1	Glycom method HPLC-703-7C7-001 and HPAEC-HMO-006
Assay (water free) – LNT	Not less than 70.0%	78	± 2	Glycom method HPLC-703-7C7-001
D-Lactose	Not more than 12.0 w/w %	7.6	± 1.5	Glycom method HPAEC-HMO-006
Lacto-*N*-triose II	Not more than 10.0 w/w %	6.4	± 1.0	Glycom method HPAEC-HMO-006

[9] It should be noted that lactose and lacto-*N*-triose II are also principal degradation products of LNT.
[10] "LNT fructose isomer" denotes an isomer of LNT where the terminal glucose sugar isomerised to a fructose sugar.

Table 2.3.1-1 Specifications for LNT

Para-LNH2	Not more than 3.5 w/w %	2.8	± 0.3	Glycom method HPAEC-HMO-006
LNT fructose isomer	Not more than 1.0 w/w %	0.7	± 0.1	Glycom method HPLC-703-7C7-001
Sum of other carbohydrates	Not more than 5.0 w/w %	1.3	± 0.1	Glycom method HPLC-703-7C7-001 and HPAEC-HMO-006
pH (20°C, 5% solution)	4.0 to 6.0	4.4	± 0.2	Ph. Eur. 9.2 2.2.3 (07/2016:20203)
Water	Not more than 6.0 w/w %	1.9	± 0.5	Glycom method KF-001
Ash, sulphated	Not more than 0.5 w/w %	0.10	± 0.03	Ph. Eur. 9.2 2.4.14 (04/2010:20414)
Residual protein by Bradford assay	Not more than 0.01 w/w %	< 0.0017		Glycom method UV-001
Lead	Not more than 0.1 mg/kg	< 0.1		EN 13805:2002 and EPA-6020A:2007
Microbiological Parameters[b]				
Aerobic mesophilic total plate count	Not more than 500 CFU/g	< 10		ISO 4833-1:2014
Enterobacteriaceae	Absent in 10 g	Complies		ISO 21528-1:2004, ISO 21528-2:2004
Salmonella spp.	Absent in 25 g	Complies		ISO 6579:2006
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Complies		ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	Complies		ISO 11290-1:1996/A1:2005
Bacillus cereus	Not more than 50 CFU/g	< 10		ISO 7932:2005
Yeasts	Not more than 10 CFU/g	< 10		ISO 7954:1999
Moulds	Not more than 10 CFU/g	< 10		ISO 7954:1999
Residual endotoxins	Not more than 10 EU/mg	0.02	± 0.02	Ph. Eur. 2.6.14

AVE = average; CFU = colony-forming units; EU = endotoxin units; Ph. Eur. = European Pharmacopoeia; HiMS = Human-identical milk saccharides (LNT + lactose + lacto-*N*-triose II); HPAEC = high-performance anion exchange chromatography; IC = ion chromatography; ISO = International Organization for Standardization; KF: Karl-Fischer; MPN = most probable number; RT= retention time.

[a]HiMS = Sum of LNT + Lactose + Lacto-*N*-triose II.

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

2.3.2 Product Analyses

2.3.2.1 Main products and Other Carbohydrates

LNT manufactured by Glycom can be described as white to off-white amorphous powder or agglomerate. Amorphous powders do not possess defined melting points. LNT is readily soluble in aqueous solutions (max. 400 mg/mL, 25°C), with poor solubility in any organic solvents. The summary of batch results corresponding to selected physicochemical properties of LNT is presented in Table 2.3.2.1-1.

Table 2.3.2.1-1 Batch Results for Selected Physicochemical Properties of LNT Product

Parameters	**Manufacturing Batch Numbers:**							
	CPN4215 1000216 FD	**CPN4215 1000316 FD**	**CPN4215 1000416 FD**	**CPN4215 1000516 FD**	**CPN4215 1000616 FD**	**AVE**	**±**	**SD**
Appearance	Powder or agglomerates							
Colour	White to off white							
pH (20°C, 5% solution)	4.6	4.0	4.3	4.5	4.4	4.4	±	0.2

2.4.1 Bulk Stability

2.4.1.1 Real-Time Stability

The bulk stability of the LNT produced from fermentation, as described herein, was investigated under real-time conditions [25°C, 60% relative humidity (RH)] and accelerated conditions (40°C, 75% RH). A real-time 5-year stability study and a 2-year accelerated stability study are currently ongoing on representative batches of LNT. The chemical, physical, microbiological, and sensory testing was performed in an ongoing 5-year storage study (25°C, 60% RH) on 2 representative batches (No. CPN4215 00115 and CPN4215 1000516 FD), with interim results available up to 24 and 9 months, respectively, at the time of filing (see Table 2.4.1.1-1). The results further confirm that the ingredient is stable when stored at ambient room temperature for at least 24 months.

Table 2.4.1.1-1 Results of the 5-Year Real-Time Stability Study on LNT Product (25°C, 60% Relative Humidity, RH) for 2 Representative Batches: A) Batch No. CPN4215 00115, and B) Batch No. CPN4215 1000516 FD

A) Manufacturing Batch Number CPN4215 00115

Parameter	Sample Time (Months)						
	0	**3**	**6**	**9**	**12**	**18**	**24**
Physical Properties							
Color	White	White	White	White	Ivory white	White	Ivory white
Appearance	Non-agglutinated powder	Agglutinated powder	Agglutinated powder	Agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder
Purity							
Water content [%]	13.6	11.9	12.2	12.4	12.5	15.6	13.0
Assay – LNT [%]	78.3	79.8	79.6	77.9	77.3	78.2	76.3
Lactose [%]	1.74	1.89	1.86	1.91	1.94	1.26	1.2
Lactulose [%]	n.r.	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Lacto-N-triose II [%]	4.0	4.33	4.11	4.68	4.79	3.25	3.4
Para-LNH2 [%]	0.68	0.66	0.67	0.65	0.66	0.78	0.8
LNT fructose isomer [%]	0.38	0.35	0.31	0.39	0.36	0.33	0.4
Assay (water free) HiMS [%]	97.3	97.6	97.5	96.4	96.0	97.9	93.0
Microbiological Quality							
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested
Salmonella spp.	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Not tested
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested
Listeria monocytogenes	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Not tested
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested

B) Manufacturing Batch Number CPN4215 1000516 FD

Parameter	Sample Time (Months)						
	0	3	6	9	12	18	24
Physical Properties							
Color	White	n.a.	n.a.	n.a.	Not tested	Not tested	Not tested
Appearance	Fine powder	n.a.	n.a.	n.a.	Not tested	Not tested	Not tested
Purity							
Water content [%]	2.4	2.2	1.8	2.2	Not tested	Not tested	Not tested
Assay – LNT [%]	74.9	76.3	76.9	73.4	Not tested	Not tested	Not tested
Lactose [%]	8.7	8.9	9.30	8.8	Not tested	Not tested	Not tested
Lactulose [%]	0.2	0.2	0.2	0.2	Not tested	Not tested	Not tested
Lacto-N-triose II [%]	5.7	5.5	5.6	5.6	Not tested	Not tested	Not tested
Para-LNH2 [%]	2.1	2.0	2.3	2.2	Not tested	Not tested	Not tested
LNT fructose isomer [%]	1.0	0.9	0.9	1.0	Not tested	Not tested	Not tested
Assay (water free) HiMS [%]	91.5	92.7	93.5	89.7	NA	NA	NA
Microbiological Quality							
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Salmonella spp.	Absent in 25 g	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Listeria monocytogenes	Absent in 25 g	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	Not tested	Not tested	Not tested

CFU = colony-forming units, LNT = lacto-*N*-tetraose, HiMS = human-identical milk saccharides = sum of LNT, lactose and lacto-*N*-triose II; NA = not applicable; n.a. = not available; n.r. = not reported; para-LNH2 = para-lacto-*N*-hexaose-2.

2.4.1.2 Accelerated Stability

The bulk stability of spray dried, amorphous LNT products was investigated, under accelerated conditions (40°C, 75% RH) for a period of 2 years. The results for 2 representative batches No CPN4215 00115 and No CPN4215 1000516 FD are presented in Table 2.4.1.2-1 below. The results of these studies indicate that there are no changes in organoleptic properties of LNT, no appreciable degradation of LNT, no changes in impurity profile, and no alterations in the microbiological quality of the ingredient following storage for up to 2 years under defined, accelerated storage conditions. LNT was analysed by HPLC and water content was analysed by Karl Fischer titration at each time point.

Two independent lots of LNT were demonstrated to be stable throughout the 24- and 9-month storage periods respectively (for Batch Nos. CPN4215 1000516 FD and CPN4215 1000516 FD, respectively) with no measurable loss of LNT, other carbohydrates or change in impurities content. As with the real-time stability testing, no appreciable changes, degradation of the ingredient, or alterations in impurity profiles were reported. Based on the results of the accelerated stability study and using the Arrhenius equation (Peleg *et*

al., 2012), the stability of the ingredient was calculated to be at least 5 years when protected from light and stored at room temperature and ambient humidity.

Table 2.4.1.2-1 Representative Interim Results of the 2-Year Accelerated Stability Study on LNT Product (40°C, 75% Relative Humidity, RH) for 2 Representative Batches: A) Batch No. CPN4215 00115, and B) Batch No. CPN4215 1000516 FD

A) Manufacturing Batch Number CPN4215 00115

Parameter	Sample Time (Months)								
	0	**1**	**2**	**3**	**6**	**9**	**12**	**18**	**24**
Physical Properties									
Colour	White	White	White	White	White	White	Ivory white	Ivory white	Ivory white
Appearance	Not agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder	Agglutinated powder	Agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder
Purity									
Water content [%]	13.6	11.7	12.7	11.8	13.1	13.0	13.5	11.4	13.4
Assay – LNT [%]	78.3	77.0	77.5	78.0	78.0	78.6	78.3	77.7	76.3
Lactose [%]	1.74	1.89	1.83	1.96	1.90	1.94	2.07	1.88	1.90
Lactulose [%]	n.r.	< 0.1	< 0.1	< 0.1	< 0.1	0.10	< 0.1	< 0.1	< 0.1
Lacto-*N*-triose II [%]	4.00	4.52	4.42	4.46	4.13	4.58	4.82	3.91	3.50
Para-LNH2 [%]	0.68	0.72	0.61	0.68	0.65	0.65	0.67	0.73	0.70
LNT fructose isomer [%]	0.38	0.39	0.36	0.37	0.34	0.37	0.44	0.38	0.40
Assay (water free) HiMS [%]	97.3	94.5	95.4	95.8	96.7	97.9	98.5	94.2	94.3
Microbiological Quality									
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Salmonella spp.	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Absent in 10 g	Not tested	Absent in 10 g
Listeria monocytogenes	Absent in 25 g	Not tested	Not tested	Not tested	Absent in 25 g	Not tested	Absent in 25 g	Not tested	Absent in 25 g
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	< 10	Not tested	< 10

B) Manufacturing Batch Number CPN4215 1000516 FD

Parameter	Sample Time (Months)								
	0	1	2	3	6	9	12	18	24
Physical Properties									
Colour	White	White	White	White	White	White	Not tested	Not tested	Not tested
Appearance	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Fine powder	Not tested	Not tested	Not tested
Purity									
Water content [%]	2.4	2.3	2.3	2.2	1.8	2.0	Not tested	Not tested	Not tested
Assay – LNT [%]	74.9	75.0	75.7	77.7	76.9	74.4	Not tested	Not tested	Not tested
Lactose [%]	8.7	8.6	9.1	9.2	9.0	8.2	Not tested	Not tested	Not tested
Lactulose [%]	0.2	0.2	0.2	0.2	0.2	0.2	Not tested	Not tested	Not tested
Lacto-*N*-triose II [%]	5.7	5.9	5.8	5.6	5.6	5.6	Not tested	Not tested	Not tested
Para-LNH2 [%]	n.a.	2.0	2.0	2.0	2.2	2.0	Not tested	Not tested	Not tested
LNT fructose isomer [%]	1.0	1.0	0.8	1.0	0.9	1.0	Not tested	Not tested	Not tested
Assay (water free) HiMS [%]	91.5	91.6	92.8	94.6	93.2	90.0	NA	NA	NA
Microbiological Quality									
Aerobic mesophilic total plate count [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Enterobacteriaceae	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Salmonella spp.	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Listeria monocytogenes	Absent in 10 g	Not tested	Not tested	Not tested	Absent in 10 g	Not tested	Not tested	Not tested	Not tested
Bacillus cereus [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Yeasts [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested
Moulds [CFU/g]	< 10	Not tested	Not tested	Not tested	< 10	Not tested	Not tested	Not tested	Not tested

CFU = colony-forming units, LNT = lacto-*N*-tetraose, HiMS = human-identical milk saccharides = sum of LNT, lactose and lacto-*N*-triose II; NA = not applicable; n.a. = not available; n.r. = not reported; para-LNH2 = para-lacto-*N*-hexaose-2.

2.4.1.3 Stress/Forced Stability

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

Forced stability tests of the bulk LNT product in aqueous solutions were performed at 60 and 80°C for 8 and 4 weeks of storage, respectively, using dissolved:

- Amorphous LNT powder at slightly acidic pH (4.5)
- Crystalline LNT powder at neutral pH (6.3) as reference
- Amorphous LNT powder at neutral pH (6.8)

The results of this study showed the presence of 2 potential pH-dependent chemical degradation pathways in the aqueous solutions of LNT product, namely hydrolysis at pH < 5.0 and isomerisation at pH > 6.0. At

From:	Marta Hanna Miks
To:	Morissette, Rachel
Subject:	RE: follow-up question for GRN 000833
Date:	Wednesday, July 17, 2019 9:15:26 AM
Attachments:	image004.png image020.png FDA Response to GRN 0008833 - July 16"19.pdf

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Dear Dr. Rachel,

Please find attached our response letter to the questions on GRN 000833 (lacto-*N*-tetraose, LNT).

And please don't hesitate to get back to us in case of any further questions or clarifications needed.

With kindest regards,
Marta

Marta H. Miks, DSc, PhD
Regulatory & Scientific Affairs Manager

_____
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Kogle Allé 4
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Tlf +45 5037 2222
mhm@glycom.com
www.glycom.com

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Wednesday, July 10, 2019 9:03 PM
To: Marta Hanna Miks <mhm@glycom.com>
Subject: FW: follow-up question for GRN 000833

Dear Ms. Miks,

I received an out-of-office message for Dr. Röhrig. Would you be able to respond to my email below?

Thank you,

Rachel Morissette, Ph.D.
Regulatory Review Scientist

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety

U.S. Food and Drug Administration
rachel.morissette@fda.hhs.gov

From: Morissette, Rachel
Sent: Wednesday, July 10, 2019 2:59 PM
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: follow-up question for GRN 000833

Dear Christoph,

Our chemist has two follow-up questions for GRN 000833. Once we have that information we can continue the review of your GRAS notice.

1. *For infant formula uses, Glycom notes that LNT may be added together with other human-identical milk oligosaccharides (HiMOs) such as LNnT and 2'-FL with target concentrations based on levels found in human milk. While we would not expect Glycom to project all possible combinations of HiMOs in infant formula, it remains unclear if these oligosaccharide uses are substitutional, partially substitutional, or largely additive. Please indicate which, if any, HiMO uses are substitutional or if they are all potentially additive. Since you have noted that the LNT ingredient will likely be used in combination with one or more related HiMOs, please provide one or more examples of combinations that would be consistent with your GRAS conclusions for use of these ingredients (GRN 000833 and previous HiMOs in GRNs 547, 650, 659) in infant formula.*

2. *For conventional foods uses, please clarify whether the uses of LNT would be substitutional (or additive) for related ingredients and similar food uses that have been the subject of previous GRNs submitted by Glycom. If LNT is likely to be used in combination with one or more related HiMOs, please provide one or more examples of combinations that would be consistent with your GRAS conclusions for use of these ingredients (GRN 000833 and previous HiMOs in GRNs 547, 650, 659) in conventional foods.*

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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


FDA
U.S. FOOD & DRUG
ADMINISTRATION

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

16 July 2019

Rachel Morissette
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotechnology and GRAS Notice Review
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740-3835 USA

Re: GRAS Notice No. GRN 000833

Dear Dr. Morissette,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s email received on 10 July 2019 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) Notice for lacto-N-tetraose (LNT) filed by the Agency under GRN 833.

***FDA.1.** For infant formula uses, Glycom notes that LNT may be added together with other human-identical milk oligosaccharides (HiMOs) such as LNnT and 2'-FL with target concentrations based on levels found in human milk. While we would not expect Glycom to project all possible combinations of HiMOs in infant formula, it remains unclear if these oligosaccharide uses are substitutional, partially substitutional, or largely additive. Please indicate which, if any, HiMO uses are substitutional or if they are all potentially additive. Since you have noted that the LNT ingredient will likely be used in combination with one or more related HiMOs, please provide one or more examples of combinations that would be consistent with your GRAS conclusions for use of these ingredients (GRN 000833 and previous HiMOs in GRNs 547, 650, 659) in infant formula.*

While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of human-identical milk oligosaccharides (HiMOs), such as 2'-FL, DFL, LNT, and LNnT, will be used in combination to produce infant formula products that are as compositionally representative of human breast milk as possible, taking into account natural variation. Accordingly, the use of LNT as described in GRN 833 would be GRAS when used in combination (*i.e.*, an additive manner) with all HiMO's that have GRAS status for use in infant formula. The agency is correct however, that some uses of Glycom's HiMO's in infant formula will be 100% substitutional. For example, 2'FL as described in GRN 546 would not be used in combination with other sources of 2'-FL (*e.g.*, GRN 650, 815); in these cases, the use of 2'-FL would be completely substitutional. Regardless of the particular combination there are two prevailing points underscoring the use of Glycom's HiMO's in infant formula that Glycom would like to emphasize:

1. GRAS uses of Glycom's HiMO's will produce infant formula products that are representative of levels that have been reported for human milk samples obtained from lactating women across all lactational stages taking into account natural variation.
2. Any infant formula containing a new HiMO, or new HiMO combination, will be subject to the laws and implementing regulations governing infant formula under section 412 of the Federal Food Drug and

Cosmetic Act (21 USC 350a). Accordingly, any combination of HiMO whether used singularly, or on an additive basis with various HiMO's will be the subject of safety and tolerance testing in infants; such information would provide corroborating evidence in support of Glycom's view that HiMO's when used in combinations that are representative of levels in human milk will be safe and well tolerated.

***FDA.2.** For conventional foods uses, please clarify whether the uses of LNT would be substitutional (or additive) for related ingredients and similar food uses that have been the subject of previous GRNs submitted by Glycom. If LNT is likely to be used in combination with one or more related HiMOs, please provide one or more examples of combinations that would be consistent with your GRAS conclusions for use of these ingredients (GRN 000833 and previous HiMOs in GRNs 547, 650, 659) in conventional foods.*

Glycom is a manufacturer of HiMO ingredients, not finished food products; as such, Glycom has no control over ingredient combinations a food company decides to use with Glycom's HiMO's. Notwithstanding this point, it is anticipated that food uses of HiMO's in conventional food applications would be in keeping the uses and use levels that are GRAS for infant formula use (*i.e.*, in ratios or combinations that produce dietary exposures that are comparable to levels consumed by infants fed breast milk). For example, as shown in Table 3.2.2-2 of GRN 833, the estimated population-wide exposures to LNT from all potential food uses in conventional food products would not exceed the exposures on a mg/kg basis that have been estimated for young infants. Glycom notes that all HiMO's that have been tested to date in animal toxicology studies have been determined to be innocuous up to the highest dietary levels tested and we are not aware of any evidence to suggest that ingestion of HiMO's at any level would be unsafe outside of potential tolerance issues common to non-digestible oligosaccharides, which are self-limiting in nature. Accordingly, it is Glycom's view that LNT can be used individually or in combination with other HiMO's provided that the use levels of all ingredients respect the levels that have been concluded to be GRAS. Similar HiMO's (*e.g.*, 2'-FL *vs.* 2'-FL/DFL) would be used in a 100% substitutional manner.

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

Population Group	Age Group (Years)	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Young Infants	0 to 6 months	141	286	80.1	165	176	301
Older Infants	7 to 12 months	197	352	99.9	127	197	352
Toddlers	1 to 3	69.1	141.0	98.5	460	70.2	141.0
Children	4 to 10	27.4	56.0	98.9	980	27.7	56.4
Female Teenagers	11 to 18	11.6	26.0	94.6	568	12.3	26.4
Male Teenagers	11 to 18	14.3	27.9	98.2	569	14.6	27.9
Female Adults of Childbearing Age	19 to 40	10.0	22.1	92.9	819	10.7	22.8
Female Adults	19 to 64	9.8	22.7	92.9	1,752	10.6	23.2
Male Adults	19 to 64	10.2	23.8	92.7	1,518	11.0	24.9
Elderly	65 and up	8.1	20.4	92.1	906	8.8	20.6
Total Population	All ages	16.2	32.0	93.8	7,045	17.2	33.6

LNT = lacto-*N*-tetraose; bw = body weight; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

*Intake data expressed as wet weight of ingredient under the proposed conditions of intended use.

We hope this information adequately addresses the Agency's questions on GRN 000833, and if there is any additional information or further clarification that is required, Glycom will happy to provide such information upon request.

Sincerely,

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

From: Christoph Röhrig
To: Morissette, Rachel
Subject: RE: question for GRN 000833
Date: Friday, October 04, 2019 4:03:37 AM
Attachments: image001.png

Dear Rachel,

Please apologise the delay of my response, but since we don't directly produce to consumer, I wanted to ensure to confirm with our vendors:

I can confirm that the proposed use of LNT in infant formulas is currently limited to milk-based non-exempt formulas.

Kind regards,
Christoph

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

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christoph.roehrig@glycom.com
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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: 26. september 2019 15:51
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: question for GRN 000833

Dear Christoph,

Can you please confirm the protein source of the infant formulas in your intended use of LNT (e.g. milk-based, soy-based, etc.)?

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

From: Christoph Röhrig
To: Morissette, Rachel
Subject: RE: response letter GRN 000833
Date: Tuesday, October 08, 2019 3:06:20 AM
Attachments: image007.png

Dear Rachel,

Many thanks for sending us the FDA response letter, much appreciated.

No need to apologize for the (limited) delay, we understand that the reason for it goes back to the beginning of the year and were outside of FDA's control.

It is our continued pleasure to interact and discuss with FDA since the quality of scientific scrutiny is simply excellent.

With kind regards,
Christoph

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

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From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: 7. oktober 2019 19:36
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: response letter GRN 000833

Dear Christoph,

Please see attached the response letter for GRN 000833. I apologize for the delay. Please let me know if you have any questions.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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