Glycom A/S Kogle Allé 4 2970 Hørsholm, Denmark

GLYCOM

895

November 27, 2019

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

Dear Dr. Gaynor:

Re: GRAS Notice for Non-Crystallized Lacto-N-neotetraose (LNnT)

Glycom, a manufacturer of Human identical Milk Oligosaccharides (HiMO) has previously concluded that the use of Lacto-N-neotetraose (LNnT) as an ingredient in non-exempt term infant formula and specific conventional food and beverage products across multiple categories is GRAS on the basis of scientific procedures. Glycom's GRAS conclusion was notified to the offices of the United States Food and Drug Administration (U.S. FDA) and filed by the agency without objection under GRN 659.

Glycom has recently extended its LNnT brand portfolio to include a non-crystallized version of LNnT. A revision of the GRAS specifications for LNnT described in GRN 659 are required to accommodate the necessary modifications to the manufacturing process that entail replacement of the crystallization purification step with a combination of optimized unit operations including spray drying; these changes will reduce the energy and environmental load of the LNnT manufacturing process and reduce the cost per unit produced.

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 non-crystallized LNnT produced by a modified strain of *E. coli* K12 (DH1), is GRAS on the basis of scientific procedures, for in the same foods and at the same levels as described in GRN 659. The intended food uses of non-crystalized LNnT 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 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,

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

GLYCOM

#895

GRAS Notice for Non-Crystallized Lacto-*N*-neotetraose (LNnT)

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835
USA

Submitted by:

Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

27 November 2019

GRAS Notice for Non-Crystallized Lacto-*N*-neotetraose (LNnT)

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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*-neotetraose (LNnT), 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 LNnT as a food ingredient for addition to non-exempt term infant formula and various conventional food products, as described herein.

Signed,

2 Dec 2019

Christoph H. Röhrig, Ph.D.
Head of Regulatory 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 5037 2222
Fax: +45 4593 3968

1.2 Common Name of the Notified Substance

Lacto-*N*-neotetraose

1.3 Conditions of Intended Use

LNnT ingredients manufactured by Glycom using chemical synthesis or microbial fermentation processes have been previously been determined to be GRAS for use in non-exempt infant formula at a use level of up to 600 mg/L of the ready-to-drink or reconstituted formula as well as in selected conventional food and beverage products. These GRAS conclusions were notified to the offices of the U.S. FDA and filed by the Agency without objection under GRN 547 (chemical synthesis) and 659 (microbial fermentation) respectively. LNnT that is the subject of this notice is produced by microbial fermentation using the same recombinant strain derivative of *Escherichia coli* (*E. coli*) K12 as described in GRN 659; however, Glycom has modified the downstream manufacturing conditions described in GRN 659 to produce LNnT without a crystallization step, as a spray-dried powder rather than as a crystalline ingredient. Food uses of LNnT will be fully substitutional on a wt/wt basis to all GRAS uses of LNnT described in GRN 547 and 659. LNnT as described herein is therefore intended for use in term non-exempt infant formulas at a use level of up to 600 mg/L of the ready-to-drink or reconstituted formula. The maximum use level was based on providing a similar level of LNnT as that which occurs in mature human breast milk. LNnT also may be used in combination with other human-identical milk oligosaccharides (HiMOs) such as 2'-fucosyllactose, lacto-*N*-tetraose, 3'-sialyllactose, and 6'-sialyllactose (2'-FL; LNT; 3'-SL; 6'-SL) such that levels of each HiMOs in a finished infant formula preparation are representative of concentrations that have been measured in human milk taking into account natural variability.

While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of HiMOs, such as 2'-FL, DFL, LNT, LNnT, 3'-SL, and 6'-SL, will be used in combination to produce infant formula products that are as compositionally representative of human breast milk as possible, taking into account their natural variation. Glycom notes that any new infant formula containing a new HiMO or new HiMO combination will be subject to the laws and implementing regulations governing infant formula under Section 412 of the Federal Food, Drug, and Cosmetic Act [21 USC §350(a)]. Specifically, under Section 412(d)(1) of the Federal Food, Drug and Cosmetic Act, a manufacture of a new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula, and this must include, among other things, a description of any reformulation of the formula or change in processing of the infant formula. Accordingly, the manufacturer will need to provide the Agency with information supporting that a particular oligosaccharide combination (*e.g.*, use of 3'-SL with LNnT or other indigestible oligosaccharides such as galacto-oligosaccharides) would be well tolerated as part of the Agency's 90-day notification procedure. Section 412 therefore ensures that any combination of HiMO whether used singularly, or on an additive basis with various HiMOs will be the subject of corroborative safety and tolerance testing in infants.

LNnT also is intended for use in various conventional food and beverage products across multiple categories as described in Table 1.3-1. As discussed for infant formula uses, food uses of LNnT as described herein will be completely substitutional to GRAS uses of LNnT that has previously been concluded to be GRAS.

Table 1.3-1 Summary of the Individual Proposed Food-Uses and Use-Levels for LNnT in Conventional Food and Beverage Products and Infant Formula

Food Category	Proposed Food-Uses	RACC	Proposed Use Level (g/RACC)	Maximum Proposed Use Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	0.6	2.5
	Sports, Isotonic, and Energy Drinks	240 mL	0.14	0.58
Dairy Product Analogs	Imitation Milks	240 mL	0.14	0.58
	Non-Dairy Yogurt	225 g	0.6	2.67
Infant and Toddler Foods	Term Infant Formulas	100 mL[a]	0.06	0.60
	Toddler Formulas	100 mL[a]	0.06	0.60
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.02 to 0.68	3.0
	Other Drinks for Young Children	120 mL	0.07	0.58
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	30 g	0.6	20.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk[b]	240 mL	0.14	0.58
Milk Products	Buttermilk	240 mL	0.14	0.58
	Flavored Milk	240 mL	0.14	0.58
	Milk-Based Meal Replacement Drinks, for Weight Reduction	240 mL	0.6	2.5
	Yogurt	225 g	0.6	2.67
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.14	0.58

LNnT = lacto-*N*-neotetraose; RACC = Reference Amounts Customarily Consumed (21 CFR §101.12 – U.S. FDA, 2019a); U.S. = United States.

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

[b] Milk is a standardized food in the United States. When the milk is fortified with LNnT it will then be classified as a milk product.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the Code of Federal Regulations (CFR) (U.S. FDA, 2019b), Glycom has concluded that the intended uses of 3'-SL as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

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

Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

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

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

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

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

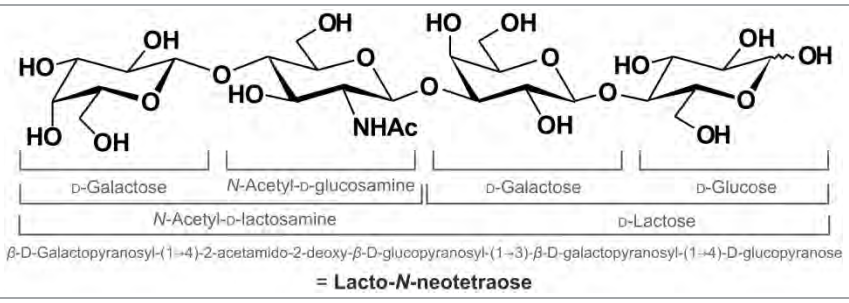
2.1 Identity

2.2 Chemical Identity

Table 2.2-1 Chemical Identity of Lacto-*N*-neotetraose

Common Name	Lacto-*N*-neotetraose
Common Abbreviation	LNnT
IUPAC Name	β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Alternative Denotations	Gal-β-(1→4)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc *N*-Acetyl-D-lactosamine-β-(1→3)-D-lactose
CAS Registry Number	13007-32-4
Chemical Formula	$C_{26}H_{45}NO_{21}$
Molecular Weight	707.63

Table 2.2-1 Chemical Identity of Lacto-*N*-neotetraose

Structural Formula	

CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry.

2.3 Chemical and Physical Characteristics

LNnT is a naturally occurring tetrasaccharide detected in some mammalian milks with the highest concentrations present in human milk and is therefore typically referred to as a human milk oligosaccharide (HMO). LNnT is a chemically defined linear tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose and D-glucose, which occurs as 1 specific constitutional isomer.

The molecular structure of LNnT was elucidated by Richard Kuhn in 1962 and since then a number of publications reported detailed structure characterization by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) techniques (Kuhn and Gauhe, 1962; Strecker *et al.*, 1989; Urashima *et al.*, 2002, 2005; Landersjö *et al*., 2005). Based on ^{1}H- and ^{13}C-NMR-, mass spectrometry (MS), and high-performance liquid chromatography (HPLC) with corona charged aerosol detector data, it is confirmed that LNnT produced by microbial fermentation is chemically and structurally identical to LNnT present in human breast milk.

2.4 Method of Manufacture

LNnT described in GRN 659 is a high purity crystalline ingredient with a purity of ≥ 92%. LNnT that is the subject of this notice is produced using the same fermentation processes and recombinant production strain described in GRN 659; however, Glycom has implemented a number of unit operation optimizations to the downstream processing of LNnT that collectively allow for the omission of the crystallization step, and now intends to produce LNnT as a spray dried powder rather than a crystalline ingredient. A comparison of the original downstream manufacturing conditions and the revised manufacturing conditions for the production of Glycom's non-crystallized LNnT preparation are presented below. Where relevant, details of the production organism and other elements of the manufacturing processes that have not changed are incorporated by reference to relevant sections in GRN 659.

LNnT is manufactured in compliance with current Good Manufacturing Practices (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The raw materials from which LNnT is derived include D-lactose as a substrate, with D-glucose, D-glycerol and ammonium salts used as carbon and nitrogen sources for fermentation. The manufacturing process can be broadly divided into 2 stages: in Stage 1 (upstream processing [USP]), D-lactose is converted *via* the metabolic intermediate "lacto-*N*-triose II" to LNnT by the cellular enzymes of the LNnT production organism. In Stage 2, the downstream processing (DSP), a series of purification and isolation steps generate the final high-purity LNnT product.

2.4.1 Production Microorganism

LNnT is produced by fermentation using biosynthetic processes introduced into a recombinant strain of *E. coli* K-12 DH1. *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 sugars, then transformed with a high-copy plasmid carrying 2 enzymes necessary for LNnT synthesis. Detailed information on the genotypic identity of the host organism including a description of all introduced genetic modifications enabling the productive biosynthesis of LNnT are described in Section II.B.1 of GRN 659. A discussion of the safety of the donor genes and corresponding expression products is presented in Section IV.G of GRN 659.

2.4.2 Manufacturing Stage 1: Fermentation Procedure

The manufacturing process for LNnT can be broadly divided into 2 stages and include upstream processes (Stage I) relating to the fermentation and biosynthesis of LNnT as well as downstream processes (Stage 2), which are largely characterized as process for purification of the ingredient.

In Stage 1 of the manufacturing process, D-lactose is converted by the production organism into LNnT by cell fermentation. The fermentation is maintained for several days until In-Process Controls indicate a favorable ratio LNnT to other carbohydrates and high consumption of D-lactose. LNnT is excreted into the fermentation broth and the microbial biomass containing the production organism is then removed from the culture supernatant containing LNnT by ultrafiltration/ diafiltration and the separated microbial biomass is deactivated by heat treatment. The quality of the clear ultrafiltration/diafiltration permeate is assessed by a range of In-Process Controls and then further purified by the second stage of the production process, the downstream processing. Detailed information on the fermentation procedures including descriptions of the raw materials are incorporated by reference to Section II.B.2 through II.B.4 of GRN 659.

2.4.3 Manufacturing Stage 2: Purification and Isolation

As discussed, changes to the manufacture of LNnT as described in GRN 659 *vs*. LNnT as described in this notice relate to implementation of manufacturing changes to produce a spray-dried product that does not require crystallization. Omission of the crystallization step has a significant impact on the technical state-of-the-art since a crystallization operation comes at a significant premium on cost, waste management (solvents) and through-put of the manufacturing line.

Glycom notes that omission of the crystallization step is feasible due to the implementation of optimized unit operations upstream in the purification sequence (which improve the performance of each purification step) accompanied with freeze or spray-drying, that will be used instead of crystallization to isolate LNnT during downstream processing. The improved purification performance of the initial unit operations has been achieved by gradual modifications to process parameters (*e.g.*, adapted process time, adapted ratio of processing aid to product stream, adapted temperature and pH), that do not change the overall process.

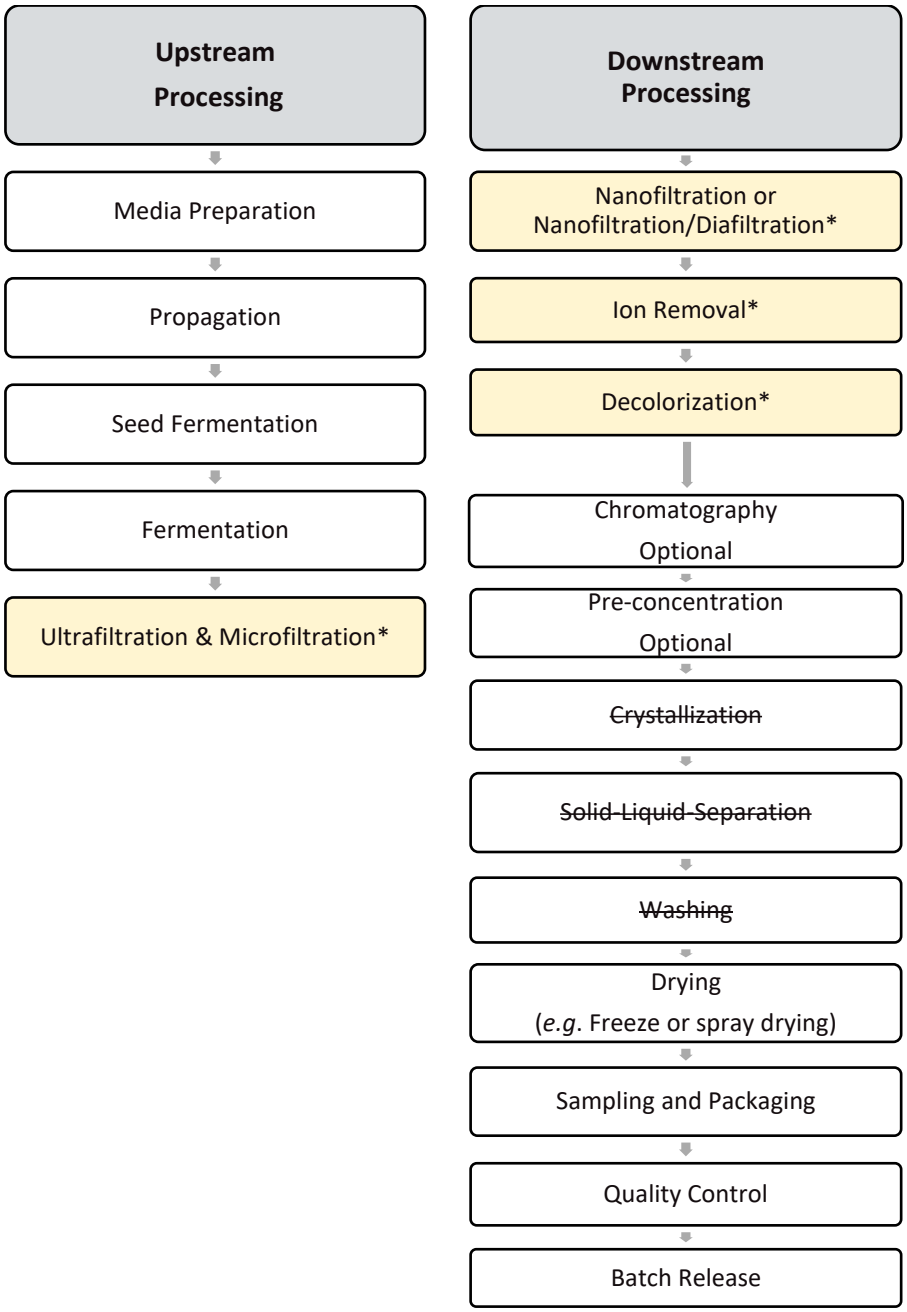
Since crystallization has been removed, a previously mandatory chromatography process has now become optional, since chromatography was previously needed to reduce the *para*-LNnH levels ahead of crystallization (increased levels of *para*-LNnH would inhibit crystallization). Thus, in combination the change eliminates the use of organic solvents in the production of LNnT (*i.e.*, methanol used for crystallization or acetic acid used in regeneration of chromatographic columns). Therefore, the change not only has an impact on the overall costs of the process, but also has a tremendous impact from an environmental perspective, as it eliminates the use of approximately 8 L of methanol and 3 kg of acetic acid per kg of produced LNnT, and therefore significantly reduces waste associated with HiMO manufacturing and also produces a finished product that is absent solvent residues.

The primary impact of omitting the crystallization step is the production of LNnT with a slightly higher level of LNnT biosynthesis related carbohydrates, namely D-lactose (≤10.0%) and para-lacto-N-neohexaose (para-LNnH; ≤5.0%), which compares to LNnT that is crystallized where lower levels of D-lactose (≤3.0%) and para-LNnH (≤3.0%) are obtained. D-Lactose and para-LNnH are naturally present in human milk, and therefore are not considered as an "impurity" as such in the LNnT ingredient.

Glycom has obtained analytical data demonstrating that the use of its improved purification procedures in lieu of crystallization largely limits the changes to D-lactose and *para*-LNnH and does not alter the levels of other metabolites (*e.g.*, residual protein, DNA, endotoxins), a conclusion that is attributed to the fact that several purification steps for removal of these compounds (*e.g.*, ion exchange and de-colorization) are maintained.

As discussed in more detail in Part 6 of this notice there are no safety concerns related to the aforementioned purity differences since the differences in purity are predominantly limited to safe and structurally related carbohydrate components. An overview of the revised downstream manufacturing processes for production of LNnT is presented below in Figure 2.4.3-1.

Figure 2.4.3-1 Overview of the Downstream Manufacturing Process for Lacto-*N*-neotetraose



* The mandatory purification steps of improved performance have been highlighted in yellow. Steps with strikethrough represent process operations applied for production of the crystalized ingredient.

2.4.4 Quality Control

The manufacture of LNnT by microbial fermentation is consistent with Good Manufacturing Practice and HACCP principles.

Due to the principal raw materials and the final product being single, well-characterized and pure compounds the whole production process can be followed in detail by a range of analytical techniques. These techniques are applied either as in-process controls or at batch release (by certificate of analysis) to allow full control of the production process.

Both manufacturing stages are controlled by a HACCP plan which includes specifications for the equipment, raw materials, product, and packaging materials used in the manufacturing process. Master operating instructions are followed, batch records are kept, a number of in-process controls are applied, and the final isolated LNnT product is controlled by certificates of analyses and batch release routines.

2.5 Specifications and Product Analysis

The compositional changes resulting from modifications in the production process are largely limited to increased levels of lactose and *para*-lacto-*N*-neohexaose (*para*-LNnH), which consequently lead to a proportional decrease of LNnT. Accordingly, the specification for LNnT has been revised to reflect these compositional changes (Table 2.5-1). All methods of analysis are either internationally recognized or developed internally by Glycom. The ingredient is specified as a white to off-white powder or agglomerates with a purity of at least 80% based on HPLC with a corona charged aerosol detector (HPLC-cCAD). Upper limits have been established for the raw materials used in the manufacturing (*e.g.,* D-lactose), the carbohydrates formed during the fermentation (*e.g.*, lacto-*N*-triose II, *para*-lacto-*N*-neohexaose, LNnT fructose isomer), heavy metals, and microbiological parameters, to ensure the purity of the final product.

Microbiological specifications have been revised from those described in GRN 659 to bring the ingredient in alignment with updated specifications that consider the use of Glycom's HiMOs during wet blending of infant formula manufacturing. Glycom has established separate specification parameters for LNnT depending on whether the ingredient is utilized in wet-blending or dry-blending of the infant formula production process. The wet-blending stage of manufacturing involves a heat-treatment step in which these microorganisms would be killed. Heat-treatments at temperatures above 75°C for 30 seconds will provide a reduction in excess of 10 log units of vegetative microorganisms such as *Salmonella* spp. or *Enterobacteriaceae*, including *Cronobacter sakazakii*; heat-treatments above 100°C will lead to reductions in excess of several hundred log units (WHO, 2006). Microbial specifications used for wet-blending applications will therefore be compliant with the microbial requirements for infant formula as defined under 21 CFR §106.55 (U.S. FDA, 2019c).

There are no significant changes to the test methods used by Glycom for the assessment of the specification parameters in their LNnT preparation as compared to GRN 659.

Table 2.5-1 Specification and Batch Analyses for Non-Crystallized Lacto-*N*-neotetraose

Parameter	GRAS Specifications		Batch Analyses					
	GRN 659	Specification Non-crystallized LNnT	AVG ± SD	NK2018_34_1	NK2018_37_1	NK2018_40_1	NK2018_46_1	NK2018_48_1
Appearance	Powder or agglomerates	Powder	Complies	Complies	Complies	Complies	Complies	Complies
Color	White to off white	White to off white	Complies	Complies	Complies	Complies	Complies	Complies
Identification	RT Standard ± 3%	RT Standard ± 3%	Complies	Complies	Complies	Complies	Complies	Complies
Assay (water-free) Human-identical Milk Saccharides[a]	*Not specified*	**≥92%**	96.0% ± 1.3%	97.2%	95.1%	97.3%	94.3%	96.1%
• LNnT (water-free)	≥92%	**≥80%**	89.1% ± 3.5%	90.9%	87.7%	93.4%	83.9%	89.4%
• D-Lactose	≤3.0%	**≤10.0%**	3.1% ± 2.1%	1.6%	4.2%	1.3%	6.3%	2.1%
• Lacto-*N*-triose II	≤3.0%	≤3.0%	0.6% ± 0.2%	0.5%	0.8%	0.5%	0.6%	0.8%
• *para*-LNnH	≤3.0%	**≤5.0%**	2.4% ± 1.0%	3.5%	1.4%	1.2%	2.7%	3.0%
• LNnT fructose isomer	≤1.0%	≤1.0%	<0.6%	<0.60%	0.64%	<0.60%	<0.60%	<0.60%
pH (20°C, 5% solution)	4.0 to 7.0	4.0 to 7.0	5.5 ± 0.7	5.7	6.0	5.8	5.8	4.2
Water	≤9.0%	≤9.0%	3.88% ± 1.42%	2.61%	5.41%	4.89%	2.18%	4.31%
Ash, sulphated	≤0.4%	≤0.6%	0.04% ± 0.07%	0.02%	0.16%	<0.08%	<0.02%	<0.01%
Methanol	≤100 mg/kg	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
Isopropanol	≤200 mg/kg	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]
Residual proteins	≤0.01%	≤0.01%	Complies	0.0036%	<LOQ[c]	<LOQ[c]	<LOQ[c]	<LOQ[c]
Microbiological Criteria[d]								
Aerobic mesophilic bacteria total count	≤500 CFU/g	**Not more than 1,000 CFU/g**	Complies	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Yeasts	≤10 CFU/g	**Not more than 100 CFU/g**	Complies	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Molds	≤10 CFU/g	**Not more than 100 CFU/g**	Complies	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Enterobacteriaceae	Absent in 10 g	**Not more than 10 CFU/g**	Complies	<100 CFU/g	<100 CFU/g	<100 CFU/g	<100 CFU/g	<100 CFU/g

Table 2.5-1 Specification and Batch Analyses for Non-Crystallized Lacto-*N*-neotetraose

Parameter	GRAS Specifications		Batch Analyses					
	GRN 659	Specification Non-crystallized LNnT	AVG ± SD	NK2018_34_1	NK2018_37_1	NK2018_40_1	NK2018_46_1	NK2018_48_1
Salmonella	Absent in 25 g	**Absent in 25 g**	Complies	Absent	Absent	Absent	Absent	Absent
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	**NA[e]**	NA[e]	NA[e]	NA[e]	NA[e]	NA[e]	NA[e]
Listeria monocytogenes	Absent in 25 g	**NA[e]**	NA[e]	NA[e]	NA[e]	NA[e]	NA[e]	NA[e]
Bacillus cereus	Not more than 50 CFU/g	**NA[e]**	NA[e]	NA[e]	NA[e]	NA[e]	NA[e]	NA[e]
Residual endotoxins	≤10 EU/mg	≤10 EU/mg	0.0522 ± 0.11	0.0002 EU/mg	0.0040 EU/mg	0.0030 EU/mg	0.2500 EU/mg	0.0040 EU/mg

AVG = average; CFU = colony-forming units; EU = endotoxin units; LOQ = limit of quantitation; NA = not applicable; SD = standard deviation.

[a] Assay (water-free) Human-identical Milk Saccharides = LNnT, D-Lactose, lacto-N-triose II, para-LNnH, LNnT fructose isomer

[b] Methanol was used as a processing aid during the crystallization step of the production process described in GRN 659. Since Glycom is now using improved purification together with freeze or spray-drying to isolate LNnT, the use of methanol is no longer required.

[c] LOQ = 0.0017% (w/w).

[d] The microbial specifications listed represent minimum requirements for LNnT that is added to infant formula and toddler formula products during the wet-mix stage of the infant formula manufacturing process, and also is suitable for conventional food products used by the general population (*i.e.,* non-infant formula and toddler formula food products). The minimum microbial requirements for LNnT that is added during the dry-blending stage of infant formula manufacturing include the following additional parameters: *Cronobacter (Enterobacter) sakazakii* (Absent in 10 g), *Listeria monocytogenes* (Absent in 25 g), and *Bacillus cereus* (not more than 50 CFU/g).

[e] Not applicable when LNnT is added to infant formula during wet blending and where subsequent heat pasteurization is applied to the formula prior spray-drying.

2.5.1 Additional Quantitative and Qualitative Analyses

The main changes applied to the production process relative to those described for LNnT in GRN 659 involve optimization of the purification steps during downstream processing, accompanied with another isolation and drying procedure (*e.g*., freeze or spray-drying) instead of the crystallization step. Accordingly, previous optional unit operations (*e.g*., ion-exchange purification) have become mandatory. Removal of the intact production microorganism occurs early in the process during upstream processing, and all other purification steps of the downstream processing (*e.g.*, filtration, decoloration) remain in place. As such, the residual levels of the production microorganism or its metabolites (protein, miner (or its metabolites) remaining in the final LNnT preparation will not be affected by this change in the production process. Data and information characterizing the quantitative and qualitative purity of LNnT are therefore incorporated by reference to Sections II.C.4 of GRN 659.

2.6 Stability

Aside from the minor changes in the levels of some of the existing saccharides in Glycom's LNnT preparation (*e.g.*, an increase in lactose and *para*-LNnH content), the modification to the processing method (as described above) does not affect the structural/chemical identity of LNnT itself. As such, no changes are expected with regard to the stability profile of Glycom's LNnT obtained from a genetically modified strain of *E. coli* K-12, neither during bulk storage nor when incorporated into food matrices.

Part 3 § 170.235 Dietary Exposure

Glycom has not changed the intended uses of LNnT relative to those previously described for the crystalized ingredient described in GRN 659. Dietary exposures to LNnT from uses in infant formula and conventional food products are therefore incorporated by reference to Section IV.A of GRN 659.

The use of LNnT as described herein will be completely substitutional to the GRAS uses of LNnT described in GRN 659, therefore introduction of the non-crystalized form of LNnT to the U.S. marketplace will not change dietary exposures to LNnT. 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, difucosyllactose (DFL), LNT, LNnT, 3'-SL and 6'-SL will be used in combination to produce infant formula products that are as compositionally representative of human breast milk as possible, taking into account their natural variation. As discussed in detail previously, in Glycom's view, GRAS uses of individual HiMOs in infant formula should be representative of levels that have been reported for human milk samples obtained from lactating women across all lactational stages considering natural variation. Consequently, the maximum level of HiMOs used in combination (*i.e*., an additive manner) in infant formula should not exceed mean quantities of total HMOs that have been measured in pooled samples of human breast milk (Kunz *et al*., 1999, 2000).

Part 4 § 170.240 Self-Limiting Levels of Use

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

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

Not applicable.

Part 6 § 170.250 Narrative and Safety Information

As described previously in GRN 659, the addition of LNnT to infant formula and conventional food products at specified use levels has been concluded to be GRAS on the basis of scientific procedures. This GRAS conclusion was notified to the offices of the U.S. FDA and filed without objection by the Agency under GRN 659. Data and information supporting the safety of LNnT are therefore incorporated by reference to Section IV of GRN 659.

Glycom has implemented several revisions to the manufacturing process, which have resulted in slight changes to the specification of LNnT described in GRN 659. The predominant change in Glycom's production process compared to that described previously in GRN 659 is the omission of crystallization, which has become feasible due to the implementation of optimized unit operations upstream in the purification sequence (which improve the performance of each purification step) accompanied with freeze or spray-drying steps that will be used instead of crystallization to isolate LNnT during downstream processing. The improved purification performance of the initial unit operations has been achieved by gradual modifications to process parameters (*e.g.*, adapted process time, adapted ratio of processing aid to product stream, adapted temperature and pH), that do not change the overall process.

Changes to the specifications for LNnT are summarized in Table 2.5-1 above. Namely, the proposed changes are justified as follows:

- The increase in the specification limit for lactose from 3.0 to ≤10% will not have any impact on the safety of LNnT, given that lactose is already a major component of milk/dairy, breast milk, and infant formula, and any intake of lactose from its presence in LNnT would be negligible in comparison.

- The increase in the specification limit for *para*-LNnH from 3.5 to ≤5% will not have an impact on the safety of LNnT. The *para*-LNnH is a naturally occurring component of breast milk, and also constitutes 1 of the core structures of HMOs, *i.e*., an intermediate HMO structure of the biosynthesis of further modified HMOs during lactation, and is thus, an integral component of more complex HMOs present in milk (*e.g*., difucosylated-*para*-LNnH and trifucosylated-*para*-LNnH).

- Due to the increased content of lactose and *para*-LNnH, the level of LNnT is reduced accordingly and Glycom proposes to specify it at ≥ 80.0%.

- However, as an additional measure of purity and reflecting that Glycom's LNnT preparation still represents a highly purified ingredient, a new specification limit of ≥92% for the sum of specified saccharides (*i.e*., LNnT, lactose, lacto-*N*-triose II, *para*-LNnH, and LNnT fructose isomer) is applied.

- Additionally, to harmonize the microbiological criteria across all of the company's HiMOs, and to consider the use of this ingredient during the wet blending stage of infant formula manufacturing, the following microbiological criteria were established: aerobic mesophilic bacteria total count ≤3,000 CFU/g, yeasts count ≤100 CFU/g and molds count ≤100 CFU/g, *Enterobacteriaceae* absent in 10 g, *Salmonella* absent in 25 g.

Glycom has presented analytical data, which demonstrated that no significant change in the composition of the ingredient occurred as a result of the new production processes that would affect its nutritional value, metabolism or level of undesirable substances when compared to the LNnT that has GRAS status under GRN 659. The proposed specification limit to increase the *para*-lacto-*N*-neohexaose (≤5%) and lactose (≤10%) limits with corresponding decrease in LNnT content (≥80%) are not of safety concern as all of the compounds are well known safe carbohydrates: *para*-LNnH and LNnT are oligosaccharides, while lactose is disaccharide, all of them are naturally present in human breast milk and have a long history of consumption by humans. The amount of lactose that would result from the intake of LNnT would be minimal compared to the amount that is already consumed through dietary sources (*e.g.*, dairy, breast milk, infant formula). Moreover, the quantity of lactose in certain food products containing LNnT, namely infant formula, are strictly defined, and the final product formulation can be easily adjusted to compensate for any increases in the lactose content of the LNnT ingredient.

The production microorganism used for the synthesis of Glycom's non-crystallized LNnT ingredient is the same production strain that is used to manufacture LNnT described in GRN 659. Since LNnT is secreted into the culture medium, and the production strain itself is removed intact during upstream processing (Stage 1), the modifications made to the final downstream processing steps (improved purification process instead of crystallization) will not have any impact on transfer of components of the production organism (*e.g.*, protein) to the finished product. This is supported by analytical data demonstrating the absence of the production strain and residual protein (*i.e.*, below the limit of quantitation of 0.0017%) in the final LNnT ingredient.

For the purposes of identifying any new data relevant to the safety of LNnT published since Glycom's previous GRAS determination (GRN 659), a comprehensive search of the published scientific literature was conducted in March 2016. The search was conducted using the electronic search tool, ProQuest, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS reviews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. A discussion of all newly available published and unpublished studies, both favorable and unfavorable, is presented below.

6.1 Absorption, Distribution, Metabolism, and Excretion (ADME)

The powder properties (amorphous *vs*. crystalline) of the LNnT ingredient are of no relevance to its use in infant nutrition products since the production of most infant beverage formulations includes dissolution, heat-treatment and spray-drying during manufacturing of the food/beverage, turning any crystalline component into an amorphous powder. Moreover, as spray-dried and crystalline LNnT have the same solubility characteristics, there will be no difference in the ADME profile of the ingredients. Apart from powder properties, the proposed specification varies only slightly in the ratio between the same major compositional constituents, like lactose, and the amount of lactose that would result from the intake of LNnT would be minimal compared to the amount that is already consumed through dietary sources (*e.g.*, dairy, breast milk, infant formula).

For a comprehensive discussion on the ADME profile of LNnT the reader is directed to Section IV.D of GRN 659.

6.2 Toxicological Studies

The general recognition of safety of LNnT under the specified conditions of use in term infant formula and conventional food and beverage products is largely based on published studies characterizing the concentrations of LNnT in human milk (see Section IV.B of GRN 659), the corresponding history of safe consumption of LNnT by breast-feeding infants, and upon data demonstrating that LNnT is of high purity and is chemically equivalent to human LNnT.

The results of published and unpublished toxicological studies in neonatal and mature rats further corroborate the safety of the ingredient. Comprehensive discussions of the published toxicity studies as they apply to the safety of LNnT for use in infant formula and foods are incorporated by reference to Section IV.B.5 of GRN 547 Section IV.E of GRN 659. These studies included a 90-day oral toxicity study in neonatal rats, as well as *in vivo* and *in vitro* genotoxicity assays. Findings from these studies demonstrated that LNnT is not genotoxic and is of low toxicity potential following gavage dosing in neonatal pups. The absence of adverse or toxicity effects reported in the literature on LNnT in animal models is consistent with its natural presence in human milk at appreciable levels. No new animal toxicology studies were identified from Glycom's updated literature.

6.3 Other Studies

Glycom's updated literature search identified limited new information from various investigational studies evaluating putative biological roles/effects of LNnT in human nutrition. Comstock *et al*. (2017) evaluated the effects of HMOs and prebiotic oligosaccharides on immune cell populations from non-infected and rotavirus-infected pigs. The study was conducted in 50 male and female piglets removed from the sow immediately after birth to avoid the ingestion of colostrum. Piglets were randomized to 1 of 3 groups administered the following milk replacer formulas: 1) Commercial milk-replacer formula; 2) milk replacer formula with 4 g of HMOs/L (40% 2'-fucosyllactose, 35% LNnT, 10% 6'-siallylactose, 5% 3'-siallylactose and 10% free sialic acid); 3 milk replacer formula with 4 g of prebiotics/L (3.6 g short chain galactooligosaccharides, and 0.4 g of long chain fructooligosaccharides. All pigs were passively immunized with sow serum. At postpartum day 10 half of the pigs were gavaged with $5x10^6$ focus forming units of porcine rotavirus. Blood samples were collected on day 5 post infection and subjected to phenotypic characterization and the enzyme-linked ImmunoSpot assay was used for identification of interferon-gamma (IFN-γ) producing cells. The authors reported that regardless of rotavirus infection, HMO-fed pigs had roughly double the number of peripheral blood mononuclear cell (PBMC) natural killer (NK) cells, 36% more mesenteric lymph node (MLN) and effector memory T cells, and 5 times more PBMC basophils. HMO fed piglets had more IFN-γ producing PBMCs and MLN cells as formula fed non-infected pigs. Macrophages and plasmacytoid and mature dendritic cell numbers were higher in the formula fed pigs than the HMO fed pigs. Overall, the authors reported that dietary HMO's were more effective than prebiotics in altering the gastrointestinal immune cells in the pigs, suggesting that the complex oligosaccharides in human milk affects infant immune development and improves neonatal response to infection. Glycom notes that the significance of the findings from this study are difficult to interpret as the study is not validated for its relevance to humans/infant development; however, the overall findings were considered nutritionally beneficial and

are consistent with the nutritional role of HMOs in human milk and the putative evolutionary nutritional role that HMOs have played in supporting infant health.

Thongaram *et al*. (2017) reported findings from an *in vitro* study evaluating the metabolism of various HMOs by 12 *Bifidobacterium* and 12 *Lactobacillus* populations. Probiotic strains were inoculated into semidefined *de Man, Rogosa and Sharpe* (MRS) broth containing 1% of various HMOs (including LNnT) or their constituent monomeric sugars. The authors reported that only *Bifidobacterium infantis* (*B. infantis*) and *Bifidobacterium breve* were able to ferment LNnT. Among the lactobacilli, *Lactobacillus acidophilus* was the most efficient at utilizing LNnT, and that this species utilized an extracellular *beta*-galactosidase to cleave the terminal galactose of LNnT leaving Lacto-N-triose II in the media. Glycom noted that findings from this study are consistent with the generally recognized biological role of HMOs as energy sources promoting the selective growth of specific microbial populations within the gastrointestinal tract following consumption from human milk or infant formula supplemented with HMOs.

Özcan and Sela (2018) reported inefficient metabolism of LNnT by *B. infantis*. Growth of *B. infantis* was measured using a microplate growth assay whereby *B. infantis* was inoculated into MRS media containing 2% glucose, lactose, LNT or LNnT as the sole carbon source. The authors reported that LNnT utilization induced a shift in the ratio of secreted acetate to lactate of 1.7 to 2.0, which compared to that observed with its component sugars (lactose and glucose) of *ca*. 1.5. The inefficient metabolism of LNnT shunted carbon synthesis pathways towards increased formic acid and ethanol secretion. Although the authors were unable to speculate on any physiological implications of carbon shunt towards formic acid and ethanol production, anti-inflammatory effects were observed on Caco-2 cells cultured in the presence of cell-free supernatants of *B. infantis* LNnT fermentation. Glycom notes that growth of select *B. infantis* strains in the presence of supraphysiological concentrations of LNnT as the sole carbon source for the organism represents experimental conditions that are not relevant to the situation *in vivo* where infants consume infant formula supplemented with HMOs. The study by Özcan and Sela was therefore not relevant to the safety assessment of LNnT as the experimental conditions are grossly artifactual relative to the *in vivo* situation.

Vazquez *et al*. (2017) evaluated the absorption of human oligosaccharides after oral administration in rats. In this study LNnT was administered to Sprague-Dawley rats (n=8 rats per dose) 8 to 10 weeks of age *via* gavage at volumes providing doses of 0.2 or 1 g/kg body weight. After gavage blood was sampled from the caudal vein at 30, 60, 90, 120, 180, 240 and 300 minutes. Analysis of HMOs in serum was carried out using ultra-performance liquid chromatograph with mass spectrometry (UPLC-MS) after sample extraction. LNnT was not detected in the serum of any animals at baseline. Concentrations of LNnT in LNnT dosed animals increased within 30 minutes of LNnT administration and maximum plasma levels in region of 4 to 5 µg/mL were reached within 30 minutes. Plasma levels of LNnT declined to below 2 µg/mL by 300 minutes. LNnT was excreted in the urine of the dosed rats, with peak concentrations reported between 120- and 300-minutes post-dosing. The partial absorption of HiMOs by infants consuming breast milk has been reported previously (Goehring *et al.,* 2014); the amounts absorbed are very low and are subsequently excreted unchanged in the urine. Overall, there were no findings in this study that were inconsistent with conclusions on the GRAS status of LNnT previously discussed in GRN 659.

Overall, there was no new information from investigational studies of LNnT *in vitro* or in animal models to suggest that use of LNnT as an ingredient in infant formula or conventional foods would be unsafe.

6.4 Human Studies

6.4.1 Controlled Intervention Studies

Several clinical studies in infants administered LNnT alone or combination with 2'-FL in infant formula and 1 study in adults administered 2'-FL and/or LNnT have been reviewed previously in GRN 547 and 659 (Prieto, 2005; Elison *et al*., 2016; Puccio *et al.*, 2017). Comprehensive discussions of these studies can be found in Section IV.B.6 of GRN 547 and Section IV.F of GRN 659.

In brief, the study by Prieto (2005), evaluated the effect of LNnT in a group of 228 healthy male and female infants and toddlers between the ages of 6 to 24 months provided infant formula containing LNnT at an inclusion level 220 mg/L for 112 days. An additional control group received the same formula without LNnT. No differences in body weight or infant length were reported between the groups, and no indication of adverse effects on *Streptococcus pneumonia* colonization or abnormal ear pathologies were reported.

The study by Puccio *et al*. (2017) was conducted in 175 healthy full-term infants from 0 to 6 months of age provided a standard infant formula or infant formula supplemented with LNnT (0.5 g/L) in combination with 2'-FL (1.0 g/L). No significant differences in formula intake were reported between the test and control groups. The mean weight gain in the test group was determined to be non-inferior to the mean weight gain in the control group in both the intent-to-treat population and the per protocol population. Infants receiving the test formula did not differ from control with regard to weight, length, head circumference, body mass index (BMI) or corresponding z-scores for digestive tolerance. Mean length, head circumference, and body mass index of the infants from enrolment to 4 months of age were not statistically significantly different between the test and control groups and were comparable with the World Health Organisation (WHO) Growth Standard. The incidence of adverse events was not higher in infants fed the test formula compared to the control standard formula. Secondary endpoints from the trial by Puccio *et al*. (2017) related to the effects of 2'-FL and LNnT on the intestinal microbiota of the infants has been reported by Alliet *et al*. (2016) and Steenhout *et al*. (2016). These studies are discussed in GRN 659. Glycom notes that the results on stool and microbiota endpoints do not raise safety concerns.

The safety and tolerability of high intake levels of 2'-FL alone or in combination with LNnT was evaluated in adults by Elison *et al*. (2016). The study was a randomized, placebo-controlled, double-blind, parallel design study involving healthy adult volunteers. The participants were provided either 2'-FL or LNnT alone (at doses of 5, 10, or 20 g per day of 2'-FL or LNnT), or a combination of 2'-FL and LNnT (5, 10, or 20 g per day as the combined amount of 2'-FL and LNnT at a ratio of 2:1). A comparator group received glucose only as a placebo control. All test articles were consumed as single daily bolus doses for 2 weeks. All adverse events reported during the study were judged to be "mild" and there were no cases of premature discontinuation from the trial due to adverse events. The results support the tolerability and safety in healthy adult men and women consuming LNnT, either alone or in combination with 2'-FL, at the doses tested. However, large bolus intakes of 20 g of LNnT may represent a gastro-intestinal tolerability threshold for some individuals. Glycom noted that the results of intake estimates demonstrate that usual consumption patterns of LNnT among U.S. consumers from all proposed food uses are well below levels that may produce undesirable gastrointestinal effects.

Results of the updated literature search identified 1 new clinical trial evaluating the administration of infant formula containing LNnT in infants (Nowak-Wegryzn *et al*., 2019). The purpose of the study was to evaluate the hypoallergenicity of a novel whey-based extensively hydrolyzed infant formula containing 2'-FL and LNnT. Both test articles (2'-FL and LNnT) were manufactured by Glycom as described in GRN 650 and 659. A total of 67 infants and children between 2 and 4 months of age with confirmed cow's milk protein allergy (CMPA) were recruited from 12 sites in the United States. Infants were randomized to 1 of 2 groups administered a 100% whey-based extensively hydrolyzed formula (EHF) supplemented with 2'-FL (1.0 g/L) and LNnT (0.5 g/L) or a commercial EHF control formula (Althéra, Nestlé Health Science, Vevey, Switzerland) that has been confirmed to be hypoallergenic. The study included a preliminary double-blind placebo-controlled food challenge (DBPCFC) with the test and control formulas in blinded cross-over fashion. The first challenge session occurred within 3 to 28 days after enrolment, and the second challenge within 2 to 7 days of the first session. Any allergic signs or symptoms (cutaneous, gastrointestinal, respiratory, or cardiovascular) attributable to the challenge formula were documented, and the challenge outcome was assessed according to pre-defined pass/fail criteria for each symptom. Subjects passing both sessions then proceeded to the 1-week open challenge at home where the test formula to assess tolerance and confirm the absence of any delayed allergic reactions. Daily formula intake was measured, as well as stool frequency, color, consistency, odor, frequency of flatulence, spitting up and or vomiting, potential allergy symptoms, or other adverse events. The statistical plan of the study was validated to ensure with 95% confidence that 90% of infants with documented cow's milk allergy will not react with defined symptoms to the formula under double-blind, placebo-controlled conditions. Of the 67 infants randomized to treatment groups, 64 completed the trial. Three infants were excluded due to protocol deviations. The authors reported 1 allergic reaction in the test formula and 1 allergic reaction to the control formula. On the intention to treat analyses 63 out of 64 infants tolerated the test formula, confirming the hypoallergenicity criteria. Findings from this study are consistent with other studies evaluating the administration of 2'-FL and LNnT to infant formula, which have demonstrated that the addition of HiMOs to infant formula at levels comparable concentrations in human milk is safe, and well tolerated.

6.4.2 Observational Longitudinal Cohort Studies

Studies examining the correlation between HMO composition and infant body weight gain have been reported (Alderete *et al*., 2015; Sprenger *et al*., 2017; Larsson *et al*., 2019). For example, Alderete *et al*. (2015) reported findings from a longitudinal cohort study of 25 mother-infant dyads recruited from the University Hospital of the University of Oklahoma. Infants were breast-fed for 6 months and breast milk samples collected at 1 and 6 months of age were analyzed for HMO composition. The relations between HMO composition and growth were then evaluated using multiple linear regression. Overall, the authors reported differential findings between various HMOs and body weight gain with some HMOs being associated with higher body fat composition and other being associated with lower body fat composition. With respect to LNnT the authors reported that at 6 months each 1 µg/ml increase in LNnT was associated with a 0.03% lower body fat composition ($p<0.01$). The authors postulated that results of their study suggest the possibility that HMO composition could influence body weight gain of infants and that the observation that breast-feeding reduces the risk of developing obesity in childhood and adolescents could be related to HMO composition of human milk.

In 2017 Sprenger *et al*. conducted an open observatory, single center, longitudinal cohort study evaluating the association between selected HMOs and infant growth. The researchers collected breast milk samples at 30, 60, and 120 days post-partum from 50 mothers who gave birth to 25 female and 25 male singleton infants. The levels of 2'-FL, LNT, LNnT, 3'-SL and 6'-SL were quantified in the milk samples. Infants were then grouped based on maternal concentrations of 2'-FL in milk at Day 30. Infants in the high 2'-FL group had reported concentrations ranging from 1,880 to 2,460 mg/L and infants in the low 2'-FL group had concentrations of 12 to 42 mg/L. The concentrations of LNnT in the low 2'-FL group was between 64 to 336 mg/L and concentrations of between 124 to 434 mg/L were measured in the high 2'-FL group. The authors reported that up to 4 months of age, no significant differences in body weight, body length, body mass index and head circumference were observed between infants who consumed breast milk with low or high concentrations of 2'-FL.

Larsson *et al*. (2019) reported findings from a sub-group analysis of HMO composition and infant weight gain as part of a larger prospective observational cohort study. 13 high weight gain and 17 normal weight gain breastfed infants were recruited for the sub-group analyses and that authors collected anthropometric data on the infants at 5 and 9 months and analyzed the HMO composition of the mother's milk. The authors reported that 2'-FL was positively associated with weight velocity at 0 to 5 months and fat mass index at 5 months. In contrast, LNnT was lower in the high weight gain infant group and negatively associated with height for age Z-scores, weight velocity from 0 to 5 months and fat mass index. The authors concluded that in their small cohort study HMO concentrations in exclusively breast-fed infants were associated with excessive weight gain.

Glycom noted that finding from cohort studies are difficult to interpret based on inherent limitations of the study designs and limited capacity to control for confounding factors in these types of studies. Although there does not appear to be any consensus from these studies on the relationship between HMO composition and infant body weight composition it seems prudent that further research is collected in this area. Glycom does note that among the existing controlled studies that have evaluated the addition of HMOs to infant formula no differences in body weight composition or infant growth have been reported to date (Section 6.4.1).

6.4.3 Reviews

Vandenplas *et al*. (2018) reported findings from a review of published evidence on HMOs including findings from *in vitro* and *in vivo* studies evaluating various biological and physiological effects of these compounds as nutritive components of human milk. The authors searched PubMed from January 1990 to April 2018. The authors reported that current clinical data suggest that the addition of HMOs to infant formula was safe and well tolerated, inducing a normal growth. The authors also stated that *"HMOs are one of the major differences between cow's milk and human milk, and available evidence indicates that these components do have a health promoting benefit. The addition of one or two of these components to infant formula is safe and brings infant formula closer to human milk".*

Additional reviews on HMOs also were reviewed and similarly reported overall support for the addition of HiMOs to infant formula (*e.g*., Plaza-Diaz *et al*., 2018; Hegar *et al*., 2019).

Overall new studies and reviews of the addition of LNnT and HiMOs in general to infant formula have not identified safety concerns with the addition of these compounds to formula and are supportive of their addition to infant formula as promising innovations in infant nutrition.

6.5 Allergenicity

The production microorganism used in the manufacturing process of LNnT is identical to that previously described in GRN 659. Since LNnT is secreted into the culture medium, and the production strain itself is removed intact during upstream processing (Stage 1), the modifications made to the final downstream processing steps (improved purification process instead of crystallization) will not have any impact on its allergenicity potential. This is supported by analytical data demonstrating that no production strain or residual proteins (*i.e.*, below the limit of quantitation of 0.0017%) remain in the final LNnT ingredient obtained by Glycom through their modified production process.

6.6 General Recognition

In 2016, LNnT manufactured using microbial fermentation was concluded, by Glycom, to have GRAS status for use in non-exempt infant formula and specified conventional food and beverage products. This GRAS conclusion was based on scientific procedures using generally available data and information obtained from the peer-reviewed literature, and on consensus among a panel of experts who were qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. Glycom's GRAS conclusion was subsequently notified to the offices of the U.S. FDA and filed by the Agency without objection under GRN 659 respectively.

Glycom has recently extended its LNnT brand portfolio to include a non-crystallized version of LNnT. A revision of the GRAS specifications for LNnT described in GRN 659 are required to accommodate the necessary modifications to the manufacturing process that entail replacement of the crystallization purification step with a combination of optimized unit operations including spray drying; these changes will reduce the energy and environmental load of the LNnT manufacturing process and reduce the cost per unit produced. Specification changes included a decrease in the levels of the LNnT from ≥ 92% to ≥80%, and corresponding increases in the levels of the minor saccharides present in the novel food, namely an increase in the levels of D-Lactose from up to 3.0% to up to 10.0%, and an increase in the levels of *para*-Lacto-N-neohexaose from up to 3.0% to up to 5.0%. To ensure that the overall purity of the ingredient Glycom also has included and additional purity specification requiring the sum of the levels of Lacto-*N*-neotetraose and of the minor saccharides (D-Lactose, Lacto-*N*-triose II, *para*-Lacto-*N*-neohexaose, and Lacto-*N*-neotetraose fructose isomer) to be equal to or greater than 92.0%.

In accordance with scientific procedures, Glycom has presented data and information within this notice to support the company's conclusion that the compositional changes imparted to the ingredient as a result of the manufacturing changes do not have material significant impact on the composition in a manner that would impact the original GRAS conclusion. General consensus among qualified experts that this conclusion is appropriate is supported by recent evaluation of these manufacturing changes and revised specifications conducted by the European Commission following submission of an application for amendment of the novel food ingredient specification for LNnT in accordance with the requirements of Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods (EU, 2017). Glycom confirms that data and information presented to the European Commission in support of the specification change for LNnT are fully representative of data and information presented in this

notification. Based on this information the Commission issued the following conclusion under implementing regulation (EU) 2019 2019/1314 of 2 August 2019.

> "*The Commission considered that the requested modifications involving saccharides of the authorised novel food which are also components of the human milk while maintaining an overall high sum of those and the other minor saccharides present in the novel food, do not alter the safety considerations that supported the authorisation of the synthetic Lacto-N-neotetraose and the Lacto-N-neotetraose produced with Escherichia coli strain K-12, and therefore do not necessitate a consultation of the European Food Safety Authority*" (EU, 2019).

Based on the above, Glycom has therefore concluded that the company's GRAS conclusion is generally recognized by appropriate qualified scientific experts. LNnT as described herein may therefore be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the Code of Federal Regulations.

Part 7 § 170.255 List of Supporting Data and Information

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Viebrock, Lauren

From: Annette Lau <annette.lau@glycom.com>
Sent: Thursday, April 2, 2020 3:37 AM
To: Viebrock, Lauren
Cc: Christoph Röhrig
Subject: RE: Questions regarding GRN 895
Attachments: FDA Response to GRN 895 - 2April2020.pdf

Follow Up Flag: Follow up
Flag Status: Flagged

Dear Ms. VieBrock,

Thank you for your email. On behalf of Dr. Röhrig and Glycom, kindly find attached our response to the questions that were attached to your last email regarding GRN 000895.

If there are any further questions, please do not hesitate to let us know.

With warmest regards,
Annette

Annette Lau, M.Sc.
Senior Regulatory Affairs Manager

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Glycom A/S
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Direct Tel: +45 6043 7274
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From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Tuesday, March 24, 2020 4:55:12 PM
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: Questions regarding GRN 895

Dear Dr. Rohrig,

During our review of GRAS Notice No. 000895, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,

Lauren

Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer

Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
Tel: 301-796-7454
lauren.viebrock@fda.hhs.gov

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

2 April 2020

Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer
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 000895

Dear Dr. VieBrock,

Please see the below responses to the United States (U.S.) Food and Drug Administration (FDA)'s letter received on March 24th, 2020 pertaining to information provided within Glycom A/S (Glycom)'s Generally Recognized as Safe (GRAS) notice for non-crystallized LNnT filed by the Agency under GRN 000895.

FDA.1. *Please clarify the intended source of the infant formula protein base (e.g. milk, soy, whey) that LNnT will be added into.*

The infant formula protein base is determined by the infant formula manufacturer, and end uses of its ingredients are outside of Glycom's control; however, infant formula products to which LNnT would be added are most likely to contain partially hydrolyzed cow's milk protein as a protein base. Although in theory all of Glycom's human identical milk oligosaccharides (HiMOs), including LNnT, could be added to infant formula products containing non-milk protein bases (*e.g.*, soy protein), the current provisions of the Food Allergen Consumer Protection and Labeling Act of 2004 (FALCPA) would require the finished product manufacturer to label such products as 'contains milk'; therefore, it seems unlikely that the addition of LNnT to such products would occur.

FDA.2. *We note that the manufacturing process for LNnT uses D-lactose as a starting material. Please state whether D-lactose is derived from milk and could introduce milk proteins into the fermentation medium and/or final ingredient.*

Virtually all of Glycom's HiMOs, including LNnT, are currently produced using lactose derived from cow's milk. Based on typical concentrations of protein that can be detected in the D-lactose raw material (typically <100 ppm) used for the production of Glycom's HiMOs, it can be estimated that the fermentation medium could contain concentrations of milk protein in the range of ~10 ppm milk protein. Glycom has demonstrated that ultrafiltration, chromatography and activated carbon purification processes utilized during the manufacturing of the company's HiMOs typically result in concentrations of total protein from the fermentation media being reduced by a factor of between 8,000- to 40,000-fold. Accordingly, the maximum theoretical concentrations of milk protein that could be transferred to Glycom's HiMOs may be in the parts per billion dimension.

FDA.3. *In your notice, some of the methods of analysis used for specification parameters are developed internally by Glycom. Please provide a clarifying statement that these methods have been validated for their respective analytes.*

Glycom confirms that the company's internal methods cited within the specification for non-crystalized LNnT (Table 2.5-1 of GRN 895) are fully validated. Glycom recognizes that the validation methods were not included in Table 2.5-1; however the methods are identical to those applied to crystalline LNnT (GRN 659), and therefore can be incorporated by reference to the methods presented in Table II.C.1-1 of GRN 659.

FDA.4. *The US does not have a definition for "toddler formula." We recognize it as formula intended for 12+ months of age. However, if it is intended for babies under 12 months of age (for example, 9-18 months), then these products must follow the infant formula regulations as the intended population includes infants less than 12 months of age. Please clarify the intended population for the uses described as "toddler formula" in the notice.*

Glycom confirms that 'toddler formula' products as described in the notice represents a category of food products that are targeted to children >12 months of age. Glycom acknowledges that a formula product marketed to infants between 9 to 18 months of age would be subject to the infant formula regulations.

Glycom hopes this information adequately addresses the Agency's questions. Should there be any additional information or further clarification required, please do not hesitate to contact us.

Sincerely,

Annette Lau, M.Sc.
Senior Regulatory Affairs Manager

Glycom A/S
Kogle Allé 4, 2970 Hørsholm, Denmark
Email: annette.lau@glycom.com

Viebrock, Lauren

From: Annette Lau <annette.lau@glycom.com>
Sent: Tuesday, April 28, 2020 12:18 PM
To: Viebrock, Lauren
Cc: Christoph Röhrig
Subject: RE: Questions regarding GRN 895
Attachments: Cover Letter - GRN 895 Corrections 28Apr'20.pdf; LNnT Non-Crystallized GRN 895 - Replacement Pages 28Apr'20.pdf

Dear Ms. Viebrock,

Thank you for bringing to our attention this typographical error. You are absolutely correct, the statement was intended to read as you have stated in your email below. This error has prompted us to revisit our GRN 895 to check for further inaccuracies and inconsistencies. With our review, we have identified two additional aspects of the Notice that required clarification.

In light of this, I have prepared a covering letter detailing where we have located these errors and am attaching the corrected replacement pages. Glycom sends our apologies for the initial oversight and thank the Agency in advance for giving us the opportunity to correct our Notice.

Please let us know if you are able to consider our response and whether there are accompanying questions or concerns.

With many thanks,
Annette

Annette Lau, M.Sc.
Senior Regulatory Affairs Manager

_____
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From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: April 28, 2020 12:23 AM
To: Annette Lau <annette.lau@glycom.com>
Cc: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: RE: Questions regarding GRN 895

Dear Ms. Lau,

We have one additional question for GRN 895. In section 1.4, you state “Glycom has concluded that the intended uses of 3’-SL as described herein are GRAS on the basis of scientific procedures.” Can you please confirm that “3’-SL” was an error and that the statement was intended to read: “Glycom has concluded that the intended uses of LNnT as described herein are GRAS on the basis of scientific procedures.” Thank you.

Regards,
Lauren

From: Annette Lau <annette.lau@glycom.com>
Sent: Thursday, April 02, 2020 3:37 AM
To: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Cc: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: RE: Questions regarding GRN 895

Dear Ms. VieBrock,

Thank you for your email. On behalf of Dr. Röhrig and Glycom, kindly find attached our response to the questions that were attached to your last email regarding GRN 000895.

If there are any further questions, please do not hesitate to let us know.

With warmest regards,
Annette

Annette Lau, M.Sc.
Senior Regulatory Affairs Manager

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From: Viebrock, Lauren <Lauren.Viebrock@fda.hhs.gov>
Sent: Tuesday, March 24, 2020 4:55:12 PM
To: Christoph Röhrig <Christoph.Roehrig@glycom.com>
Subject: Questions regarding GRN 895

Dear Dr. Rohrig,

During our review of GRAS Notice No. 000895, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Regards,
Lauren

Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer

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28 April 2020

Ms. Lauren VieBrock
Consumer Safety Officer/Microbiology Reviewer
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Dear Ms. VieBrock,

Re: Questions regarding GRN 895

The Agency has recently brought to Glycom's attention a typographical error in Section 1.4 of the Generally Recognized as Safe (GRAS) Notice for non-crystallized lacto-*N*-neotetraose (LNnT) (GRN 895). Glycom regrets the error and is herein supplying a replacement page (page 5) now correctly stating that Glycom has concluded that the intended uses of LNnT are GRAS on the basis of scientific procedures.

In response, Glycom took the opportunity to review the contents of the Notice for accuracy and consistency. We hereby note additional corrections are required with respect to the description of downstream technologies employed in the manufacture of LNnT.

1. With respect to the drying of the ingredient, standard drying operations such as freeze- or spray-drying may be utilized in the manufacture of LNnT. This is indicated in the manufacturing flow chart and is described in Sections 2.4.3, 2.5.1, and Part 6; however, it is inconsistently reflected in the remaining Sections of the Notice. Glycom wishes to clarify this point by enclosing replacement pages 4, 7, 8, 16, 22, now correctly signifying that drying is not limited to spray-drying.
2. With respect to the "ion-exchange purification" step, Glycom herein notes that this is not a precise scientific description of the actual technology applied in the purification of this ingredient and should be more accurately described as ion adsorption filtration. Replacement pages 9 and 14 are now duly enclosed.

Glycom would also note here that no changes in the manufacture of LNnT have been implemented; rather, the Notice now more accurately describes the operations being employed.

Glycom apologizes for the oversight in the initial submission of this Notice and remains at the Agency's disposal should there be any questions or concerns regarding these corrections.

Best Regards,

Annette Lau, M.Sc.

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1.3 Conditions of Intended Use

LNnT ingredients manufactured by Glycom using chemical synthesis or microbial fermentation processes have been previously been determined to be GRAS for use in non-exempt infant formula at a use level of up to 600 mg/L of the ready-to-drink or reconstituted formula as well as in selected conventional food and beverage products. These GRAS conclusions were notified to the offices of the U.S. FDA and filed by the Agency without objection under GRN 547 (chemical synthesis) and 659 (microbial fermentation) respectively. LNnT that is the subject of this notice is produced by microbial fermentation using the same recombinant strain derivative of *Escherichia coli* (*E. coli*) K12 as described in GRN 659; however, Glycom has modified the downstream manufacturing conditions described in GRN 659 to produce LNnT without a crystallization step, as a dried powder rather than as a crystalline ingredient. Food uses of LNnT will be fully substitutional on a wt/wt basis to all GRAS uses of LNnT described in GRN 547 and 659. LNnT as described herein is therefore intended for use in term non-exempt infant formulas at a use level of up to 600 mg/L of the ready-to-drink or reconstituted formula. The maximum use level was based on providing a similar level of LNnT as that which occurs in mature human breast milk. LNnT also may be used in combination with other human-identical milk oligosaccharides (HiMOs) such as 2'-fucosyllactose, lacto-*N*-tetraose, 3'-sialyllactose, and 6'-sialyllactose (2'-FL; LNT; 3'-SL; 6'-SL) such that levels of each HiMOs in a finished infant formula preparation are representative of concentrations that have been measured in human milk taking into account natural variability.

While Glycom is not a manufacturer of infant formula, the company anticipates that their portfolio of HiMOs, such as 2'-FL, DFL, LNT, LNnT, 3'-SL, and 6'-SL, will be used in combination to produce infant formula products that are as compositionally representative of human breast milk as possible, taking into account their natural variation. Glycom notes that any new infant formula containing a new HiMO or new HiMO combination will be subject to the laws and implementing regulations governing infant formula under Section 412 of the Federal Food, Drug, and Cosmetic Act [21 USC §350(a)]. Specifically, under Section 412(d)(1) of the Federal Food, Drug and Cosmetic Act, a manufacture of a new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula, and this must include, among other things, a description of any reformulation of the formula or change in processing of the infant formula. Accordingly, the manufacturer will need to provide the Agency with information supporting that a particular oligosaccharide combination (*e.g.*, use of 3'-SL with LNnT or other indigestible oligosaccharides such as galacto-oligosaccharides) would be well tolerated as part of the Agency's 90-day notification procedure. Section 412 therefore ensures that any combination of HiMO whether used singularly, or on an additive basis with various HiMOs will be the subject of corroborative safety and tolerance testing in infants.

LNnT also is intended for use in various conventional food and beverage products across multiple categories as described in Table 1.3-1. As discussed for infant formula uses, food uses of LNnT as described herein will be completely substitutional to GRAS uses of LNnT that has previously been concluded to be GRAS.

Table 1.3-1 Summary of the Individual Proposed Food-Uses and Use-Levels for LNnT in Conventional Food and Beverage Products and Infant Formula

Food Category	Proposed Food-Uses	RACC	Proposed Use Level (g/RACC)	Maximum Proposed Use Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	0.6	2.5
	Sports, Isotonic, and Energy Drinks	240 mL	0.14	0.58
Dairy Product Analogs	Imitation Milks	240 mL	0.14	0.58
	Non-Dairy Yogurt	225 g	0.6	2.67
Infant and Toddler Foods	Term Infant Formulas	100 mL[a]	0.06	0.60
	Toddler Formulas	100 mL[a]	0.06	0.60
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.02 to 0.68	3.0
	Other Drinks for Young Children	120 mL	0.07	0.58
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	30 g	0.6	20.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk[b]	240 mL	0.14	0.58
Milk Products	Buttermilk	240 mL	0.14	0.58
	Flavored Milk	240 mL	0.14	0.58
	Milk-Based Meal Replacement Drinks, for Weight Reduction	240 mL	0.6	2.5
	Yogurt	225 g	0.6	2.67
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.14	0.58

LNnT = lacto-*N*-neotetraose; RACC = Reference Amounts Customarily Consumed (21 CFR §101.12 – U.S. FDA, 2019a); U.S. = United States.

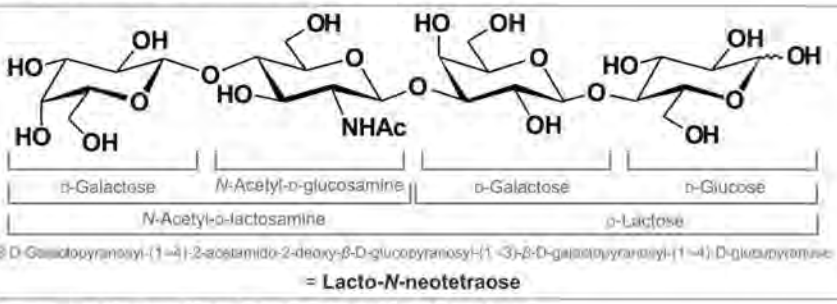
[a] RACC not available, 100 mL employed as an approximation.

[b] Milk is a standardized food in the United States. When the milk is fortified with LNnT it will then be classified as a milk product.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the Code of Federal Regulations (CFR) (U.S. FDA, 2019b), Glycom has concluded that the intended uses of LNnT as described herein are GRAS on the basis of scientific procedures.

Table 2.2-1 Chemical Identity of Lacto-*N*-neotetraose

Structural Formula	
	= **Lacto-*N*-neotetraose**

CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry.

2.3 Chemical and Physical Characteristics

LNnT is a naturally occurring tetrasaccharide detected in some mammalian milks with the highest concentrations present in human milk and is therefore typically referred to as a human milk oligosaccharide (HMO). LNnT is a chemically defined linear tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose and D-glucose, which occurs as 1 specific constitutional isomer.

The molecular structure of LNnT was elucidated by Richard Kuhn in 1962 and since then a number of publications reported detailed structure characterization by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) techniques (Kuhn and Gauhe, 1962; Strecker *et al.*, 1989; Urashima *et al.*, 2002, 2005; Landersjö *et al.*, 2005). Based on ^{1}H- and ^{13}C-NMR-, mass spectrometry (MS), and high-performance liquid chromatography (HPLC) with corona charged aerosol detector data, it is confirmed that LNnT produced by microbial fermentation is chemically and structurally identical to LNnT present in human breast milk.

2.4 Method of Manufacture

LNnT described in GRN 659 is a high purity crystalline ingredient with a purity of ≥ 92%. LNnT that is the subject of this notice is produced using the same fermentation processes and recombinant production strain described in GRN 659; however, Glycom has implemented a number of unit operation optimizations to the downstream processing of LNnT that collectively allow for the omission of the crystallization step, and now intends to produce LNnT as a dried powder rather than a crystalline ingredient. A comparison of the original downstream manufacturing conditions and the revised manufacturing conditions for the production of Glycom's non-crystallized LNnT preparation are presented below. Where relevant, details of the production organism and other elements of the manufacturing processes that have not changed are incorporated by reference to relevant sections in GRN 659.

LNnT is manufactured in compliance with current Good Manufacturing Practices (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The raw materials from which LNnT is derived include D-lactose as a substrate, with D-glucose, D-glycerol and ammonium salts used as carbon and nitrogen sources for fermentation. The manufacturing process can be broadly divided into 2 stages: in Stage 1 (upstream processing [USP]), D-lactose is converted *via* the metabolic intermediate "lacto-*N*-triose II" to LNnT by the cellular enzymes of the LNnT production organism. In Stage 2, the downstream processing (DSP), a series of purification and isolation steps generate the final high-purity LNnT product.

2.4.1 Production Microorganism

LNnT is produced by fermentation using biosynthetic processes introduced into a recombinant strain of *E. coli* K-12 DH1. *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 sugars, then transformed with a high-copy plasmid carrying 2 enzymes necessary for LNnT synthesis. Detailed information on the genotypic identity of the host organism including a description of all introduced genetic modifications enabling the productive biosynthesis of LNnT are described in Section II.B.1 of GRN 659. A discussion of the safety of the donor genes and corresponding expression products is presented in Section IV.G of GRN 659.

2.4.2 Manufacturing Stage 1: Fermentation Procedure

The manufacturing process for LNnT can be broadly divided into 2 stages and include upstream processes (Stage I) relating to the fermentation and biosynthesis of LNnT as well as downstream processes (Stage 2), which are largely characterized as process for purification of the ingredient.

In Stage 1 of the manufacturing process, D-lactose is converted by the production organism into LNnT by cell fermentation. The fermentation is maintained for several days until In-Process Controls indicate a favorable ratio LNnT to other carbohydrates and high consumption of D-lactose. LNnT is excreted into the fermentation broth and the microbial biomass containing the production organism is then removed from the culture supernatant containing LNnT by ultrafiltration/ diafiltration and the separated microbial biomass is deactivated by heat treatment. The quality of the clear ultrafiltration/diafiltration permeate is assessed by a range of In-Process Controls and then further purified by the second stage of the production process, the downstream processing. Detailed information on the fermentation procedures including descriptions of the raw materials are incorporated by reference to Section II.B.2 through II.B.4 of GRN 659.

2.4.3 Manufacturing Stage 2: Purification and Isolation

As discussed, changes to the manufacture of LNnT as described in GRN 659 *vs*. LNnT as described in this notice relate to implementation of manufacturing changes to produce a dried product that does not require crystallization. Omission of the crystallization step has a significant impact on the technical state-of-the-art since a crystallization operation comes at a significant premium on cost, waste management (solvents) and through-put of the manufacturing line.

Glycom notes that omission of the crystallization step is feasible due to the implementation of optimized unit operations upstream in the purification sequence (which improve the performance of each purification step) accompanied by a drying procedure (*e.g.* freeze or spray-drying), that will be used instead of crystallization to isolate LNnT during downstream processing. The improved purification performance of the initial unit operations has been achieved by gradual modifications to process parameters (*e.g.*, adapted process time, adapted ratio of processing aid to product stream, adapted temperature and pH), that do not change the overall process.

Since crystallization has been removed, a previously mandatory chromatography process has now become optional, since chromatography was previously needed to reduce the *para*-LNnH levels ahead of crystallization (increased levels of *para*-LNnH would inhibit crystallization). Thus, in combination the change eliminates the use of organic solvents in the production of LNnT (*i.e.*, methanol used for crystallization or acetic acid used in regeneration of chromatographic columns). Therefore, the change not only has an impact on the overall costs of the process, but also has a tremendous impact from an environmental perspective, as it eliminates the use of approximately 8 L of methanol and 3 kg of acetic acid per kg of produced LNnT, and therefore significantly reduces waste associated with HiMO manufacturing and also produces a finished product that is absent solvent residues.

The primary impact of omitting the crystallization step is the production of LNnT with a slightly higher level of LNnT biosynthesis related carbohydrates, namely D-lactose (≤10.0%) and para-lacto-N-neohexaose (para-LNnH; ≤5.0%), which compares to LNnT that is crystallized where lower levels of D-lactose (≤3.0%) and para-LNnH (≤3.0%) are obtained. D-Lactose and para-LNnH are naturally present in human milk, and therefore are not considered as an "impurity" as such in the LNnT ingredient.

Glycom has obtained analytical data demonstrating that the use of its improved purification procedures in lieu of crystallization largely limits the changes to D-lactose and *para*-LNnH and does not alter the levels of other metabolites (*e.g.*, residual protein, DNA, endotoxins), a conclusion that is attributed to the fact that several purification steps for removal of these compounds (*e.g.*, ion adsorption and de-colorization) are maintained.

As discussed in more detail in Part 6 of this notice there are no safety concerns related to the aforementioned purity differences since the differences in purity are predominantly limited to safe and structurally related carbohydrate components. An overview of the revised downstream manufacturing processes for production of LNnT is presented below in Figure 2.4.3-1.

2.5.1 Additional Quantitative and Qualitative Analyses

The main changes applied to the production process relative to those described for LNnT in GRN 659 involve optimization of the purification steps during downstream processing, accompanied with another isolation and drying procedure (*e.g.*, freeze or spray-drying) instead of the crystallization step. Accordingly, previous optional unit operations (*e.g.*, ion adsorption filtration) have become mandatory. Removal of the intact production microorganism occurs early in the process during upstream processing, and all other purification steps of the downstream processing (*e.g.*, filtration, decoloration) remain in place. As such, the residual levels of the production microorganism or its metabolites (protein, miner (or its metabolites) remaining in the final LNnT preparation will not be affected by this change in the production process. Data and information characterizing the quantitative and qualitative purity of LNnT are therefore incorporated by reference to Sections II.C.4 of GRN 659.

2.6 Stability

Aside from the minor changes in the levels of some of the existing saccharides in Glycom's LNnT preparation (*e.g.*, an increase in lactose and *para*-LNnH content), the modification to the processing method (as described above) does not affect the structural/chemical identity of LNnT itself. As such, no changes are expected with regard to the stability profile of Glycom's LNnT obtained from a genetically modified strain of *E. coli* K-12, neither during bulk storage nor when incorporated into food matrices.

Part 3 § 170.235 Dietary Exposure

Glycom has not changed the intended uses of LNnT relative to those previously described for the crystalized ingredient described in GRN 659. Dietary exposures to LNnT from uses in infant formula and conventional food products are therefore incorporated by reference to Section IV.A of GRN 659.

The use of LNnT as described herein will be completely substitutional to the GRAS uses of LNnT described in GRN 659, therefore introduction of the non-crystalized form of LNnT to the U.S. marketplace will not change dietary exposures to LNnT. 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, difucosyllactose (DFL), LNT, LNnT, 3'-SL and 6'-SL will be used in combination to produce infant formula products that are as compositionally representative of human breast milk as possible, taking into account their natural variation. As discussed in detail previously, in Glycom's view, GRAS uses of individual HiMOs in infant formula should be representative of levels that have been reported for human milk samples obtained from lactating women across all lactational stages considering natural variation. Consequently, the maximum level of HiMOs used in combination (*i.e.*, an additive manner) in infant formula should not exceed mean quantities of total HMOs that have been measured in pooled samples of human breast milk (Kunz *et al.*, 1999, 2000).

Part 4 § 170.240 Self-Limiting Levels of Use

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

- Additionally, to harmonize the microbiological criteria across all of the company's HiMOs, and to consider the use of this ingredient during the wet blending stage of infant formula manufacturing, the following microbiological criteria were established: aerobic mesophilic bacteria total count ≤3,000 CFU/g, yeasts count ≤100 CFU/g and molds count ≤100 CFU/g, *Enterobacteriaceae* absent in 10 g, *Salmonella* absent in 25 g.

Glycom has presented analytical data, which demonstrated that no significant change in the composition of the ingredient occurred as a result of the new production processes that would affect its nutritional value, metabolism or level of undesirable substances when compared to the LNnT that has GRAS status under GRN 659. The proposed specification limit to increase the *para*-lacto-*N*-neohexaose (≤5%) and lactose (≤10%) limits with corresponding decrease in LNnT content (≥80%) are not of safety concern as all of the compounds are well known safe carbohydrates: *para*-LNnH and LNnT are oligosaccharides, while lactose is disaccharide, all of them are naturally present in human breast milk and have a long history of consumption by humans. The amount of lactose that would result from the intake of LNnT would be minimal compared to the amount that is already consumed through dietary sources (*e.g.*, dairy, breast milk, infant formula). Moreover, the quantity of lactose in certain food products containing LNnT, namely infant formula, are strictly defined, and the final product formulation can be easily adjusted to compensate for any increases in the lactose content of the LNnT ingredient.

The production microorganism used for the synthesis of Glycom's non-crystallized LNnT ingredient is the same production strain that is used to manufacture LNnT described in GRN 659. Since LNnT is secreted into the culture medium, and the production strain itself is removed intact during upstream processing (Stage 1), the modifications made to the final downstream processing steps (improved purification process instead of crystallization) will not have any impact on transfer of components of the production organism (*e.g.*, protein) to the finished product. This is supported by analytical data demonstrating the absence of the production strain and residual protein (*i.e.*, below the limit of quantitation of 0.0017%) in the final LNnT ingredient.

For the purposes of identifying any new data relevant to the safety of LNnT published since Glycom's previous GRAS determination (GRN 659), a comprehensive search of the published scientific literature was conducted in March 2016. The search was conducted using the electronic search tool, ProQuest, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS reviews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. A discussion of all newly available published and unpublished studies, both favorable and unfavorable, is presented below.

6.1 Absorption, Distribution, Metabolism, and Excretion (ADME)

The powder properties (amorphous *vs*. crystalline) of the LNnT ingredient are of no relevance to its use in infant nutrition products since the production of most infant beverage formulations includes dissolution, heat-treatment and spray-drying during manufacturing of the food/beverage, turning any crystalline component into an amorphous powder. Moreover, as dried and crystalline LNnT have the same solubility characteristics, there will be no difference in the ADME profile of the ingredients. Apart from powder properties, the proposed specification varies only slightly in the ratio between the same major compositional constituents, like lactose, and the amount of lactose that would result from the intake of LNnT would be minimal compared to the amount that is already consumed through dietary sources (*e.g.*, dairy, breast milk, infant formula).

6.5 Allergenicity

The production microorganism used in the manufacturing process of LNnT is identical to that previously described in GRN 659. Since LNnT is secreted into the culture medium, and the production strain itself is removed intact during upstream processing (Stage 1), the modifications made to the final downstream processing steps (improved purification process instead of crystallization) will not have any impact on its allergenicity potential. This is supported by analytical data demonstrating that no production strain or residual proteins (*i.e.*, below the limit of quantitation of 0.0017%) remain in the final LNnT ingredient obtained by Glycom through their modified production process.

6.6 General Recognition

In 2016, LNnT manufactured using microbial fermentation was concluded, by Glycom, to have GRAS status for use in non-exempt infant formula and specified conventional food and beverage products. This GRAS conclusion was based on scientific procedures using generally available data and information obtained from the peer-reviewed literature, and on consensus among a panel of experts who were qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. Glycom's GRAS conclusion was subsequently notified to the offices of the U.S. FDA and filed by the Agency without objection under GRN 659 respectively.

Glycom has recently extended its LNnT brand portfolio to include a non-crystallized version of LNnT. A revision of the GRAS specifications for LNnT described in GRN 659 are required to accommodate the necessary modifications to the manufacturing process that entail replacement of the crystallization purification step with a combination of optimized unit operations including drying (*e.g.* freeze or spray-drying); these changes will reduce the energy and environmental load of the LNnT manufacturing process and reduce the cost per unit produced. Specification changes included a decrease in the levels of the LNnT from ≥ 92% to ≥80%, and corresponding increases in the levels of the minor saccharides present in the novel food, namely an increase in the levels of D-Lactose from up to 3.0% to up to 10.0%, and an increase in the levels of *para*-Lacto-N-neohexaose from up to 3.0% to up to 5.0%. To ensure that the overall purity of the ingredient Glycom also has included and additional purity specification requiring the sum of the levels of Lacto-*N*-neotetraose and of the minor saccharides (D-Lactose, Lacto-*N*-triose II, *para*-Lacto-*N*-neohexaose, and Lacto-*N*-neotetraose fructose isomer) to be equal to or greater than 92.0%.

In accordance with scientific procedures, Glycom has presented data and information within this notice to support the company's conclusion that the compositional changes imparted to the ingredient as a result of the manufacturing changes do not have material significant impact on the composition in a manner that would impact the original GRAS conclusion. General consensus among qualified experts that this conclusion is appropriate is supported by recent evaluation of these manufacturing changes and revised specifications conducted by the European Commission following submission of an application for amendment of the novel food ingredient specification for LNnT in accordance with the requirements of Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods (EU, 2017). Glycom confirms that data and information presented to the European Commission in support of the specification change for LNnT are fully representative of data and information presented in this