GRAS NOTICE FOR 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
5001 Campus Drive
College Park, MD
20740 USA

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

DATE:
30 June 2021

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

TABLE OF CONTENTS

List of Figures and Tables

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

Part 1. § 170.225 Signed Statements and Certification

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

Signed, 

30 June 2021

Joeri Beauprez, PhD
Chief Scientific Officer (CSO)

Date

1.1 Name and Address of Notifier

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

1.2 Common Name of Notified Substance

Lacto-*N*-neotetraose; LNnT

1.3 Conditions of Use

Inbiose's LNnT is Intended for use as an ingredient in non-exempt infant formula for term infants; in toddler beverages and other drinks for young children at a maximum level of 0.6 g/L. LNnT also is intended for use in beverages and beverage bases, dairy product analogs, milk (whole and skim), milk products, and processed fruits and juices at levels up to 2.5 g/L, in grain products, pastas, and infant and toddler foods at levels up to 20 g/kg. Use levels and food categories are incorporated by reference to Table I.D-1 of GRN 659 (replicated in Table 1.3-1 below) and are fully substitutional to those described in the Notice (GRN 659; U.S. FDA, 2016).

Table 1.3-1 Intended Food Uses and Use Levels for LNnT in the U.S. (Adapted from GRN 659)

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Uses[c]	RACC	Proposed Use Level (g/RACC)	Proposed Maximum 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[d]	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

CFR = *Code of Federal Regulations*; GRN = GRAS Notice; LNnT = lacto-*N*-neotetraose; RACC = Reference Amounts Customarily Consumed per Eating Occasion (based on values established in 21 CFR §101.12 – U.S. FDA, 2020b); 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.
[c] Food uses are identical and fully substitutional to use levels and food categories described in GRN 659.
[d] Formula/beverage products intended for toddlers >12 months of age.

1.4 Basis for GRAS

Pursuant to 21 CFR §170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2020c), Inbiose has concluded that the intended uses of LNnT as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

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

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

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

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

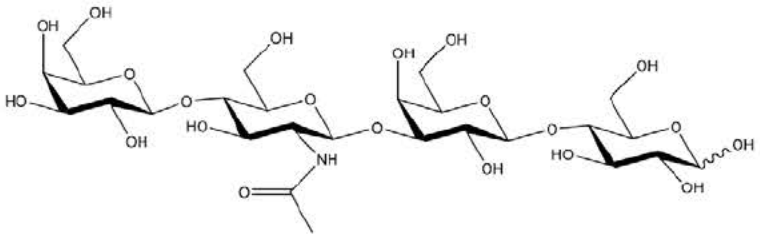
It is Inbiose's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the *Freedom of Information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

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

2.1 Identity

Common Name:	Lacto-*N*-neotetraose
Abbreviation:	LNnT
International Union of Pure and Applied Chemistry (IUPAC) Name:	beta-D-galacto-hexopyranosyl-(1->4)-2-acetamido-2-deoxy-beta-D-gluco-hexopyranosyl-(1->3)-beta-D-galacto-hexopyranosyl-(1->4)-D-gluco-hexopyranose
Chemical Abstracts Service (CAS) Number:	13007-32-4
Chemical Formula:	$C_{26}H_{45}NO_{21}$
Molecular Weight:	707.63 g/mol

Chemical structure:



Schematic representation:

2.1.1 Chemical and Physical Characteristics

LNnT is an abundant human milk oligosaccharide (HMO), comprised of galactose, glucose, and *N*-acetylglucosamine.

Inbiose's LNnT is produced by fermentation with a genetically modified strain of *Escherichia coli* K-12 MG1655. The final product is a highly purified white powder containing ≥80% LNnT, and small quantities of lactose, lacto-*N*-triose, and other related carbohydrates.

The identity of Inbiose's LNnT has been confirmed by nuclear magnetic resonance (NMR), by comparison with a LNnT reference standard (Batch ID: 45/08, IsoSep AB, Sweden) derived from human milk. Based on NMR, the Inbiose LNnT is structurally identical to the IsoSep reference. All peaks seen in the reference material are present in the Inbiose products with the same intensity. The typical shifts of the anomeric protons/carbons and those of the methyl group of the acetyl group further confirm the LNnT structure.

2.2 Manufacturing

2.2.1 Production Microorganism

2.2.1.1 Host Organism

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

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

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

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

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

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

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

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

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

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

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

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

2.2.1.2 Production Strain

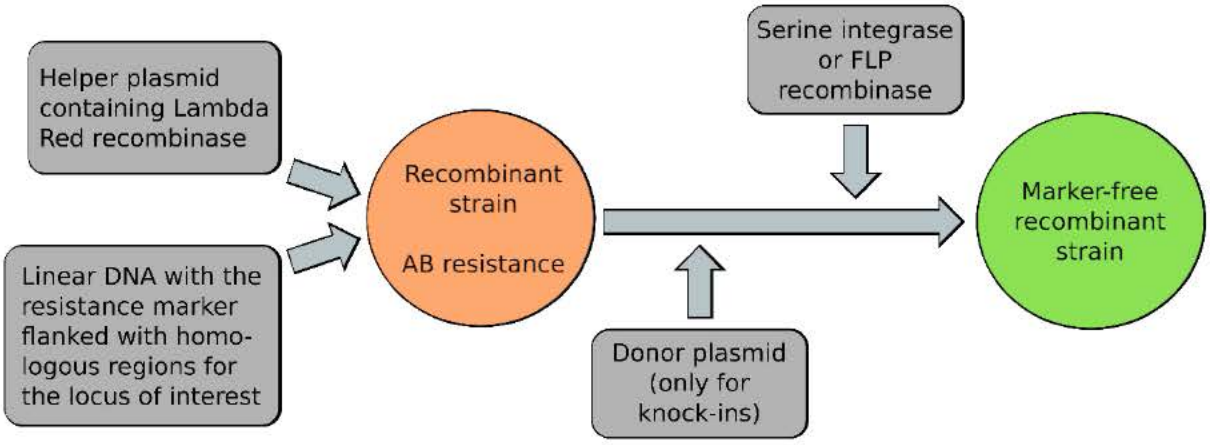
Several modifications, like gene knock-outs and gene insertions, were performed in *E. coli* K-12 strain MG1655 to create an LNnT production strain. A production strain, INB-LNnT_01, has been developed, through which the safety was assessed.

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

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

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

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



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

All heterologous genes introduced into INB-LNnT_01 were produced by DNA synthesis and were based on well-known annotated genomes from the respective donor organism. As such, no PCR techniques were used, indicating that there is no risk of undesirable or unintended genes from the donor organism being introduced to the production host. If needed, the heterologous genes were codon-optimized using bioinformatic tools. Also, before and after introducing these heterologous genes into the genome of the production host organism, a full Sanger sequencing of the transcription units was performed to ensure their identity.

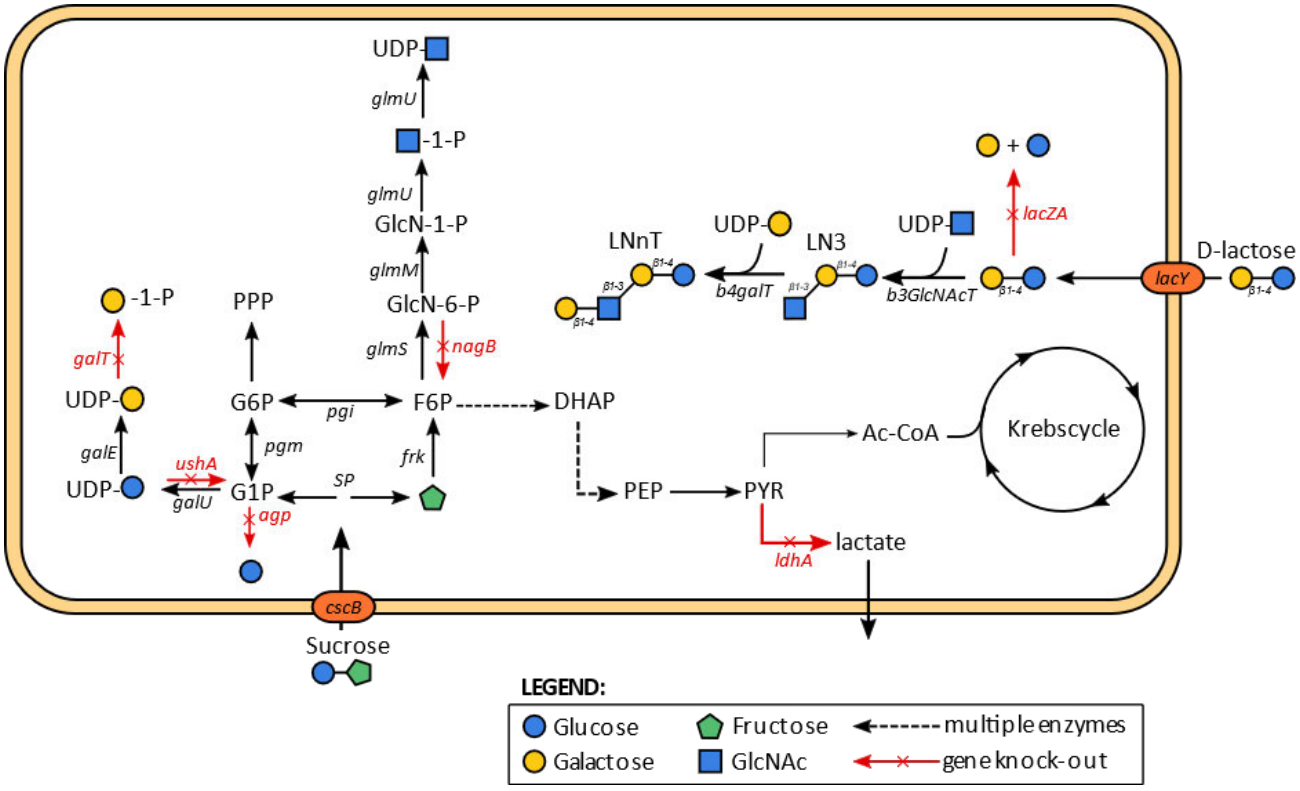
The host organism *E. coli* K-12 strain MG1655 was modified by genomic knock-outs and knock-ins by using the methods as described above to obtain efficient biosynthesis of LNnT (see Table 2.2.1.2-1 and Figure 2.2.1.2-2).

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

Origin	Function
Escherichia coli	Lactose permease
Escherichia coli	Sucrose permease
Bifidobacterium adolescentis	Sucrose phosphorylase
Zymomonas mobilis	Fructokinase
Neisseria meningitidis	beta-*N*-acetylglucosaminyltransferase
Neisseria meningitidis	beta-galactosyltransferase
Helicobacter pylori	beta-galactosyltransferase

Knock-outs were performed to avoid breakdown of lactose, improve the flux towards UDP-GlcNAc and UDP-Gal formation, and avoid the production of unwanted metabolic by-products. This strain was further modified to biosynthesize LNnT by the introduction of genes throughout the genome (see Table 2.2.1.2-1). After strain construction, colony PCR, Sanger sequencing, and WGS checks were performed to verify all genetic modifications introduced in the LNnT production strain. Production strain INB-LNnT_01 does not contain any antibiotic resistant marker on the plasmid or introduced inside its genome.

Figure 2.2.1.2-2 Schematic Overview of the LNnT Biosynthetic Pathway in INB-LNnT_01 using Sucrose as Carbon Source



Ac-CoA = acetyl-coenzyme A; DHAP = dihydroxyacetone phosphate; F6P = fructose-6-phosphate; G1P = glucose-1-phosphate; G6P = glucose-6-phosphate; GlcN-1-P = glucosamine-1-phosphate; GlcN-6-P = glucosamine-6-phosphate; LN3 = lacto-*N*-triose; LNnT = lacto-*N*-neotetraose; PEP = phosphoenolpyruvate; PPP = pentose phosphate pathway; PYR = pyruvate; UDP = uridine diphosphate.

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

Production strain INB-LNnT_01 proved to be 100% stable within the production environment after analysis by next generation sequencing of samples at the end of fermentation at pilot scale. The genes integrated into the genome cannot be mobilized or transferred by vector mediated processes such as conjugation. There are no known lytic phages or conjugation plasmids in these host strains; therefore, transfer can only occur by natural transformation. The integrated genes can be transferred at a frequency normal for chromosomal genes.

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

The production process of LNnT with INB-LNnT_01 does not require the addition of any antibiotics or inducer molecules. During the fermentation process, the production strains remain intact and convert their carbon source (sucrose) into LNnT, which is partly secreted into the medium. Afterwards, the remaining intracellular LNnT is released after pasteurization. Finally, all remaining biomass of the production hosts is removed *via* a series of downstream processing steps. As such, both production hosts are solely used as a processing aid for LNnT biosynthesis and cannot be found in the final product.

The production strain INB-LNnT_01 was deposited in an internationally recognized culture collection having acquired the status of International Depository Authority under the Budapest Treaty in Belgium.

More specifically, the strain INB-LNnT_01 with deposition number LMBP 12728 was deposited at:

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

2.2.2 Raw Materials, Processing Aids, and Equipment Specifications

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

2.2.3 LNnT Manufacturing Process

In summary, the manufacturing method for LNnT entails a fermentation process with a K-12 based production host (see Section 2.2.1) that produces LNnT. This host produces LNnT through the utilization of a carbon source (sucrose), combined with lactose in a minimal medium. The product is partly secreted into the medium. The remaining intracellular LNnT is released after pasteurization. The broth is then subjected to downstream purification and concentration processes to isolate LNnT from lactose and other structurally similar compounds as well as purification steps to remove impurities originating from fermentation (*e.g.*, minerals, proteins, and other cellular matter) followed by spray-drying (see Figures 2.2.3-1 and 2.2.3-2 below).

In the first step, biomass is removed together with cell components and large molecules (DNA, protein and lipopolysaccharides). After removal of larger particles, the majority of the salts present in the medium are removed, which are cations (*e.g.*, magnesium, calcium, ammonium) and anions (*e.g.*, phosphate and sulfate, which are minerals used for growth of the microorganism). Water is removed from the product mainly through evaporation, after which the product is polished to remove color and small amounts of residual ions. Before drying the product is filtered again to ensure the microbial specification.

Figure 2.2.3-1 Fermentation Process

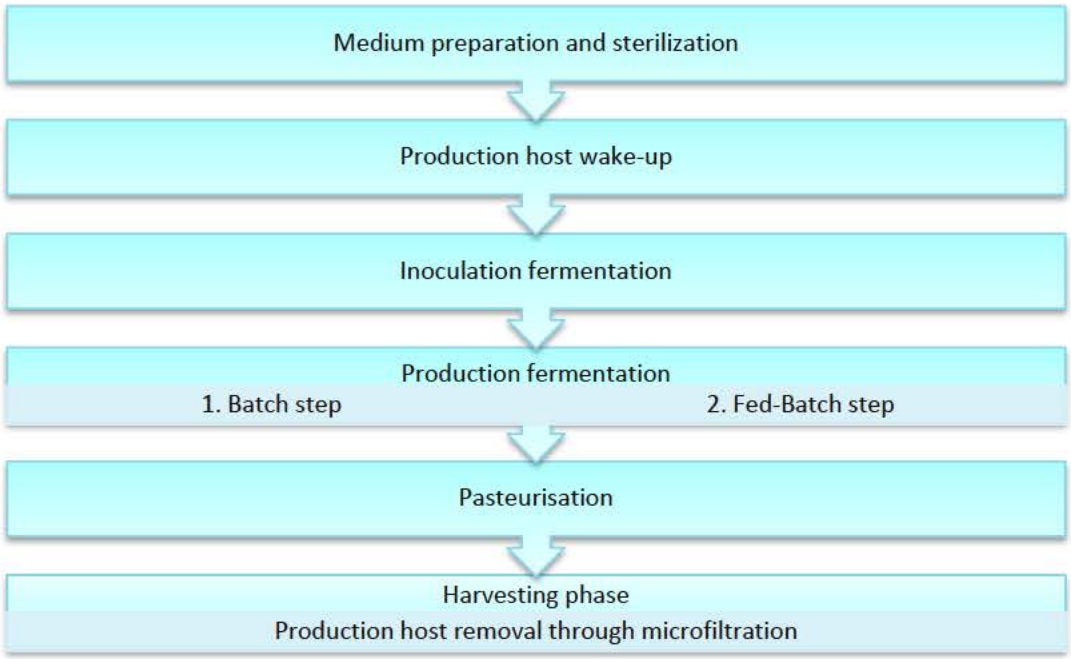
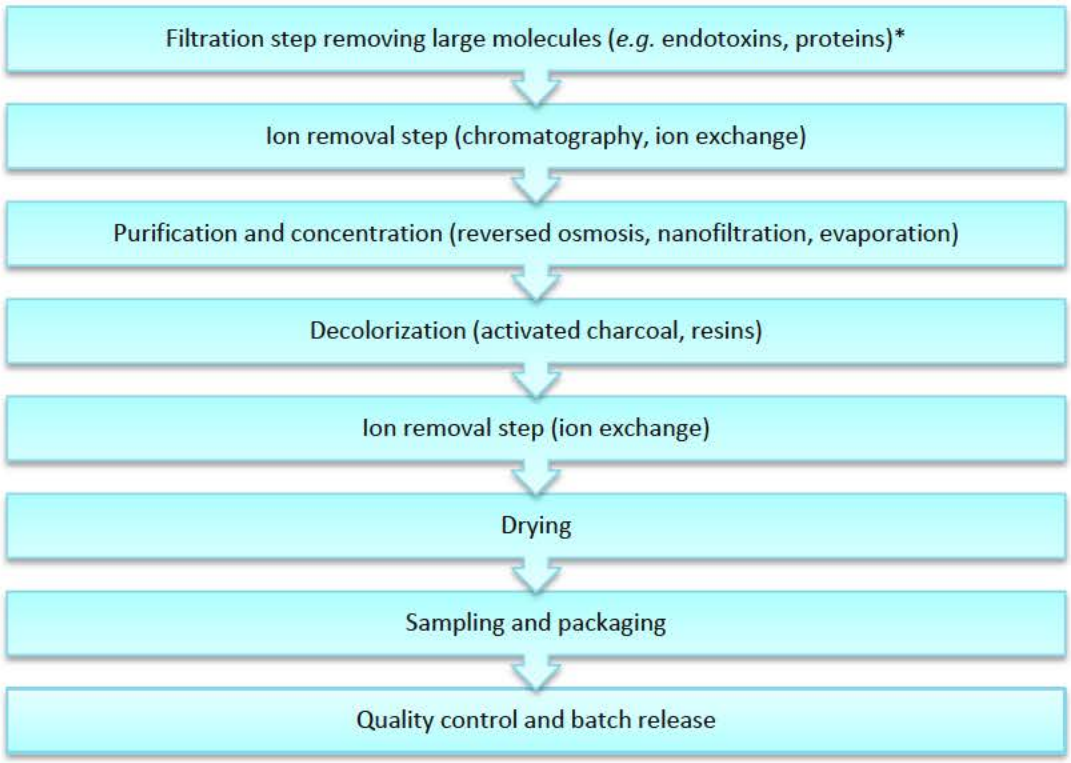


Figure 2.2.3-2 Purification Process



* The filtration steps are conducted with cut-offs of 0.1 µm to 5 µm and 1 to 30 kDa

2.3 Product Specifications and Batch Analyses

2.3.1 Specifications

To ensure consistent product quality, Inbiose has established a set of specifications for LNnT, which include the amount of LNnT and other main carbohydrates, chemical parameters, heavy metals, microbial contaminants, and absence of the genetically modified production strain and endotoxins. The specifications proposed for LNnT are presented in Table 2.3.1-1. The specifications of Glycom A/S's (Glycom's) and Jennewein Biotechnologie GmbH's (Jennewein's) LNnT preparations (GRN 547, 659, 895 and 919, respectively) are included in the table for comparison (U.S. FDA, 2014, 2016, 2020f,g). All parameters were determined using compendial or validated methods.

Table 2.3.1-1 Product Specifications for Inbiose's LNnT in Comparison to the LNnT Ingredients in GRN 547, 659, 895, and 919

Parameter	Specification for Inbiose's LNnT	Method of Analysis Employed by Inbiose	Specification Reported for Other LNnT Products			
			Glycom's LNnT from Chemical Synthesis (GRN 547)	Glycom's LNnT from Microbial Fermentation (GRN 659)	Glycom's LNnT from Microbial Fermentation, Non-crystalized (GRN 895)	Jennewein's LNnT from Microbial Fermentation (GRN 919)
Identification						
Appearance (Color)	White	Visual	White to off-white	White to off-white	White to off-white	White to off-white
Appearance (Form)	Dry powder	Visual	Powder	Powder or glomerates	Powder	Powder or glomerates
Appearance in solution	Clear, colorless to slightly yellow	Visual	NS	NS	NS	NS
Identity (LNnT)	Conform to reference standard (LNnT derived from human milk)	UPLC-RI and NMR	RT of standard ±3% (HPLC)	RT of standard ±3% (HPLC)	RT of standard ±3% (HPLC)	NMR and LC-MS
pH	4.0 to 7.0 (20°C, 10% solution)	Eurofins' internal method, potentiometry	4.0 to 7.0 (20°C, 5% solution)	4.0 to 7.0 (20°C, 5% solution)	4.0 to 7.0 (20°C, 5% solution)	5.0 to 7.0 (20°C, 5% solution)
Carbohydrates, %DM						
LNnT	NLT 80%	UPLC-RI	NLT 95.0%	NLT 92.0%	NLT 80%	NLT 92.0%
Lactose	NMT 10.0%	UPLC-RI	NS	NMT 3.0%	NMT 10.0%	NMT 3.0%
Lacto-*N*-triose (LN3)	NMT 5.0%	UPLC-RI	NS	NMT 3.0%	NMT 3.0%	NMT 3.0%
Fructo-lacto-*N*-neotetraose	NMT 1.0%	UPLC-RI	NS	NMT 1.0%	NMT 1.0%	NS
para-Lacto-*N*-neohexaose	NMT 5.0%	UPLC-RI	NS	NMT 3.0%	NMT 5.0%	NMT 2.0%
Sum of human identical milk saccharides	NLT 92%[a]	UPLC-RI	NS	NLT 95.0%[b]	NLT 92%[a]	NS
Sum of other carbohydrates	NS	UPLC-RI	NS	NS	NS	NMT 8.0%*

Table 2.3.1-1 Product Specifications for Inbiose's LNnT in Comparison to the LNnT Ingredients in GRN 547, 659, 895, and 919

Parameter	Specification for Inbiose's LNnT	Method of Analysis Employed by Inbiose	Specification Reported for Other LNnT Products			
			Glycom's LNnT from Chemical Synthesis (GRN 547)	Glycom's LNnT from Microbial Fermentation (GRN 659)	Glycom's LNnT from Microbial Fermentation, Non-crystalized (GRN 895)	Jennewein's LNnT from Microbial Fermentation (GRN 919)
Chemical Analysis						
Water content	NMT 9.0%	Karl-Fisher, volumetric	NMT 9.0%	NMT 9.0%	NMT 9.0%	NMT 9.0%
Protein content	NMT 100 µg/g	Roti®-Nanoquant	NMT 0.1%	NMT 0.01%	NMT 0.01%	NMT 100 µg/g
Ash content	NMT 0.4% w/w	NEN 6810 (500–550°C)	NMT 0.4%	NMT 1.5%	NMT 0.6%	NMT 0.4%
Acetic acid (as free acid and/or sodium acetate)	NS	-	NMT 0.3%	NS	NS	NS
Residual solvents	NS	-	NMT 50 mg/kg (singly) or 200 mg/kg (in combination)	MeOH NMT 100 mg/kg IPOH NMT 200 mg/kg	NS	NS
Residual endotoxins (IU = EU)	NMT 300 IU/g	Ph. Eur. 2.6.14	NMT 50 EU/mg	NMT 10 EU/mg	NMT 10 EU/mg	NMT 10 EU/mg
Heavy Metals						
Arsenic	NMT 0.2 mg/kg	ICP-MS	NS	NS	NS	NMT 0.2 mg/kg
Cadmium	NMT 0.1 mg/kg	ICP-MS	NS	NS	NS	NMT 0.1 mg/kg
Lead	NMT 0.05 mg/kg	ICP-MS	NMT 0.8 mg/kg	NMT 0.1 mg/kg	NS	NMT 0.02 g/kg
Mercury	NMT 0.5 mg/kg	ICP-MS	NS	NS	NS	NMT 0.5 mg/kg
Microbiological Contaminants						
Total plate count	NMT 10,000 CFU/g	ISO 4833	NMT 500 CFU/g	NMT 500 CFU/g	NMT 1,000 CFU/g	NMT 500 CFU/g
Enterobacteriaceae	Absent in 10 g	ISO 21528-2	Absent in 10 g	Absent in 10 g	NMT 10 CFU/g	NMT 10 CFU/g
Salmonella spp.	Absent in 25 g	ISO 6579-1	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Absent in 25 g	ISO/TS 22964	Absent in 10 g	Absent in 10 g	NS	Absent in 10 g
Listeria monocytogenes	Absent in 25 g	AFNOR EGS 38/05-03/17	Absent in 25 g	Absent in 25 g	NS	Absent in 25 g
Bacillus cereus	NMT 50 CFU/g	ISO 7932	NMT 50 CFU/g	NMT 50 CFU/g	NS	NMT 50 CFU/g
Yeasts	NMT 100 CFU/g	ISO 7954	NMT 10 CFU/g	NMT 10 CFU/g	NMT 100 CFU/g	NMT 100 CFU/g
Molds	NMT 100 CFU/g	ISO 7954	NMT 10 CFU/g	NMT 10 CFU/g	NMT 100 CFU/g	NMT 100 CFU/g

CFU = colony forming units; DM = dry matter; EU = endotoxin units; Glycom = Glycom A/S; GRN = GRAS Notice; HPLC = high-performance liquid chromatography; ICP-MS = inductively coupled plasma mass spectrometry; IPOH = isopropyl alcohol; ISO = International Organization for Standardization; IU = international units; Jennewein = Jennewein Biotechnologie GmbH; LC-MS = liquid chromatography–mass spectrometry; LNnT = lacto-*N*-neotetraose; MeOH = methanol; NLT = not less than; NMR = nuclear magnetic resonance; NMT = not more than; NS = not specified; Ph. Eur. = European Pharmacopoeia;

Table 2.3.1-1 Product Specifications for Inbiose's LNnT in Comparison to the LNnT Ingredients in GRN 547, 659, 895, and 919

Parameter	Specification for Inbiose's LNnT	Method of Analysis Employed by Inbiose	Specification Reported for Other LNnT Products			
			Glycom's LNnT from Chemical Synthesis (GRN 547)	Glycom's LNnT from Microbial Fermentation (GRN 659)	Glycom's LNnT from Microbial Fermentation, Non-crystalized (GRN 895)	Jennewein's LNnT from Microbial Fermentation (GRN 919)

RT = retention time; UPLC-RI = ultra-high performance liquid chromatography coupled with refractive index detector.
[a] Human identical milk saccharides is defined as the sum of LNnT, lactose, lacto-*N*-triose, fructo-lacto-*N*-neotetraose, and *para*-lacto-*N*-neohexaose.
[b] Human-identical milk saccharides was defined as the sum of LNnT, lactose, lacto-*N*-triose, and *para*-lacto-*N*-hexaose

2.3.2 Batch Analysis

Results for the analyses of 5 non-consecutive batches of LNnT are summarized in Table 2.3.2-1. The data demonstrate that the production process as described in Section 2.2 results in a consistent product that meets the established product specifications.

Table 2.3.2-1 Analytical Data Obtained from 5 Batches of LNnT

Parameter	Specification	Lot Nos.				
		ilex12F06	ilex15F02	ilex15F03	ilex15F06	ilex15F08
Identification						
Appearance (Color)	White	White	White	White	White	White
Appearance (Form)	Dry powder	Dry powder	Dry powder	Dry powder	Dry powder	Dry powder
Appearance in solution	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow
pH (20°C, 10% solution)	4.0 to 7.0	6.05	5.43	5.00	5.94	6.58
Carbohydrates, %DM						
LNnT	≥80	90.09	92.95	90.85	89.01	92.16
Lactose	≤10.0	3.27	0.00	1.13	1.54	0.00
Lacto-*N*-triose (LN3)	≤5.0	2.07	1.76	2.10	1.61	1.12
Fructo-lacto-*N*-neotetraose	≤1.0	0.00	0.41	0.76	0.57	0.50
para-Lacto-*N*-neohexaose	≤5.0	3.37	3.98	3.29	3.90	4.14
Sum of human identical milk saccharides[a]	≥92	98.80	99.10	98.13	96.63	97.92
Chemical Analysis						
Water content, volumetric (w/w %)	≤9.0	4.6	5.3	5.6	5.0	6.5
Protein content (µg/g)	≤100	<25	<25	<25	<25	<25
Ash content (%)	≤0.4	<0.1	<0.1	<0.1	<0.1	<0.1
Residual endotoxins (IU/g)	NMT 300 IU/g	3.2	1.75	3.25	0.55	63.5
Heavy Metals						
Arsenic (mg/kg)	≤0.2	<0.01	<0.01	<0.01	<0.01	<0.01
Cadmium (mg/kg)	≤0.01	<0.005	<0.005	<0.005	<0.005	<0.005
Lead (mg/kg)	≤0.05	<0.01	<0.01	<0.01	<0.01	<0.01

Table 2.3.2-1 Analytical Data Obtained from 5 Batches of LNnT

Parameter	Specification	Lot Nos.				
		ilex12F06	ilex15F02	ilex15F03	ilex15F06	ilex15F08
Mercury (mg/kg)	≤0.1	<0.01	<0.01	<0.01	<0.01	<0.01
Microbiological Contaminants						
Total plate count (CFU/g)	≤10,000	<100	<100	<100	<100	<100
Enterobacteriaceae	Absent in 10 g	Absent	Absent	Absent	Absent	Absent
Salmonella spp.	Absent in 25 g	Absent	Absent	Absent	Absent	Absent
Cronobacter (Enterobacter) sakazakii	Absent in 25 g	Absent	Absent	Absent	Absent	Absent
Listeria monocytogenes	Absent in 25 g	Absent	Absent	Absent	Absent	Absent
Bacillus cereus (CFU/g)	≤50	<10	<10	<10	<10	<10
Yeasts (CFU/g)	≤100	<10	<10	<10	<10	<10
Molds (CFU/g)	≤100	<10	<10	10[b]	<10	<10

CFU = colony forming units; DM = dry matter; IU = international units; LNnT = lacto-*N*-neotetraose; NMT = not more than.
[a] Human identical milk saccharides is defined as the sum of LNnT, lactose, lacto-*N*-triose, fructo-lacto-*N*-neotetraose, and *para*-lacto-*N*-neohexaose
[b] Estimated value (Limit of Quantification = 10 CFU/g).

2.3.3 Microbiological Endotoxins and Residual Protein Analysis

The content of endotoxins and residual proteins in the LNnT product is determined by methods with high sensitivity [Protein content: Roti®-Nanoquant method, based on the Bradford assay; and Endotoxins: kinetic-chromogenic test (Method D) described in the European Pharmacopoeia] to ensure the consistency and quality of the LNnT product.

The regulatory batches contain a low amount of endotoxin and residual proteins, meeting the proposed specifications (see Table 2.3.2-1).

2.3.4 Residual DNA Analysis

To ensure the absence of residual DNA of the production organism, PCRs were performed on the LNnT product of 5 regulatory batches of INB-LNnT_01. The protocol followed the European Food Safety Authority (EFSA) guidelines for the presence of recombinant DNA. A short subsequence of the inserted sucrose phosphorylase gene of *Bifidobacterium adolescentis* and the inserted beta-galactosyltransferase gene of *Neisseria meningitidis* was targeted to check for residual DNA in the LNnT product from all 5 batches. For every batch, the analysis was performed in triplicate together with 3 types of positive controls and 1 negative control. The analysis of all regulatory batches of LNnT showed no detectable levels of residual DNA in the final product. The limit of detection for the sucrose phosphorylase gene and the beta-galactosyltransferase gene subsequences were below the threshold limit of detection of 10 ng DNA per gram LNnT as it is stated in the EFSA guidelines (EFSA, 2018).

2.4 Stability

The stability of Inbiose's LNnT is supported by the real-time and accelerated stability studies summarized in Section II.D, page 10, of GRN 547. These studies were also used to support the stability of GRN 659 and 919. The compositional similarities between Inbiose's LNnT and the LNnT ingredients summarized in GRN 547,

659, and 919 (see Section 2.3.1), indicate that stability of the ingredients will be similar. A summary of the real-time and accelerated stability studies, as described in GRN 547 is provided below.

As described in GRN 547, the chemical and microbiological stability of Glycom's crystalline LNnT (produced by chemical synthesis) was assessed in an ongoing 5-year study under real-time conditions [25°C, 60% relative humidity (RH)]. The 36-month interim results confirmed that, when stored under ambient conditions, no significant changes were observed in the assay value for LNnT (for up to 36 months of storage) and, *N*-acetyl-lactosamine, the potential degradation product of LNnT, was not detected (following 18 months of storage), and no unknown degradation products were observed in the high-performance liquid chromatography (HPLC) chromatogram (up to 18 months of storage). Hence, the shelf-life of crystalline LNnT was determined to be 36 months when stored at ambient temperature and protected from humidity. The results of an accelerated 6-month accelerated stability study (40°C, 75% RH) also indicated no changes in the evaluated chemical and microbiological parameters. When later referenced in GRN 659, it was concluded that LNnT is stable when stored for up to 5 years under ambient storage conditions and up to 2 years under accelerated storage conditions. As the LNnT ingredient described in GRN 895 only has some minor changes in the saccharide content – which do not alter the structural and chemical identity – a stability study was not conducted.

The stability of LNnT was also assessed under the intended conditions of use. The LNnT ingredient produced by Glycom was assessed in a powdered infant formula in combination with another HiMO, 2'*O*-fucosyllactose (2'-FL), long chain polyunsaturated fatty acids (LC-PUFA), vitamins, and minerals, as described in GRN 547 and 659. The LNnT in infant formula was found to be stable up to 900 days of storage at various temperatures (4°C, 20°C, 30°C, and 37°C). The methods used to test the stability of LNnT in food applications is described in GRN 547. Briefly, the stability studies were used with formulations representative of commercial foods that were subject to standard processing (*i.e.*, pasteurization and/or ultra high-temperature heating) and storage conditions (*e.g.*, temperature and shelf-life) for these products. It was concluded that LNnT is stable when added to yogurt, citrus fruit drinks, and ready-to-drink chocolate-flavored milk following processing (as described above) and when stored at 4°C over the shelf-life of these foods.

These results indicate LNnT is anticipated to be stable in most food matrices.

Part 3. § 170.235 Dietary Exposure

3.1 Estimated Intake of LNnT (Infants and Toddlers)

3.1.1 Methods

Inbiose's LNnT is intended to be added to non-exempt term infant formula, foods targeted to infants and young children and specific used in conventional food and beverage products for the general U.S. population (see Table 1.3-1). Proposed food uses include infant formula (*i.e.,* infants up to 12 months) at concentrations up to 0.6 g/L, toddler formulas for young children >12 months of age (*e.g.*, growing up milks) at levels up to 0.6 g/L, and other beverages targeted to young children up to 0.58 g/L and up to 3.0 g/kg for products other than beverages (*e.g.,* baby foods). As food uses of LNnT are fully substitutional to current GRAS uses previously determined to be GRAS in GRN 659 no change in dietary intake of LNnT are expected from the introduction of Inbiose's LNnT ingredient to the U.S. marketplace. A summary of the estimated dietary intake of LNnT from food uses described in GRN 659 are presented below and are considered applicable to GRAS uses of LNnT described herein.

3.1.2 Intake Estimates for LNnT

3.1.2.1 Infant Formula

As described in GRN 659, the estimated daily intake of LNnT as an ingredient in term infant formula (0 to 12 months) and toddler formula has been estimated from dietary survey data. The intake of LNnT described in GRN 659 was estimated using food categories representative of each proposed food use chosen from the National Center for Health Statistics' 2009-2010 National Health and Nutrition Examination Survey (NHANES) (CDC, 2011; USDA, 2012). Infants (0 to 6 months) were established to have the greatest all-user estimated mean and 90th percentile intake of LNnT at 0.51 and 0.73 g/person/day, respectively. The summary of the estimated dietary intake of LNnT from use in infant formula in the infant and toddler population, as described in GRN 659, is provided in Table 3.1.2.1-1 (U.S. FDA, 2016).

Table 3.1.2.1-1 Summary of the Estimated Daily Intake of LNnT from Infant Formulas in the U.S. Infant and Toddler Population Groups (2009-2010 NHANES Data)[a]

Population Group	Age Group (Years)	All-Person Consumption (g/day)		All-Users Consumption (g/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Infants	0 to 6 months	0.38	0.68	74.8	161	0.51	0.73
Infants	7 to 12 months	0.31	0.62	73.6	128	0.42	0.66
Toddlers	1 to 3	<0.01[b]	na	1.1	7	0.27[b]	0.35[b]

GRN = GRAS Notice; LNnT = lacto-*N*-neotetraose; na = not available; NHANES = National Health and Nutrition Examination Survey.
[a] Table adapted from GRN 659 (U.S. FDA, 2016), full intake assessment reported in GRN 659 GRAS determination.
[b] Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements.

3.1.2.2 Infant Formula and Food Combined

The estimated intake of LNnT as an ingredient from its use in term infant formulas combined with its use in foods was also considered in order to calculate the total daily intake of the ingredient from all sources in the population groups identified as consumers of infant formula (*i.e.*, infants and toddlers). The intake of LNnT described in GRN 659 was estimated using food categories representative of each proposed food use chosen from the National Center for Health Statistics' 2009-2010 NHANES (CDC, 2011; USDA, 2012). Due to the high percentage of identified users, only the all-user results are presented herein. The greatest contributors to the estimated daily intakes of LNnT were use in infant formulas and use in baby foods for infants and young children. Infants (7 to 12 months) were established to have the greatest all-user estimated mean and 90th percentile intake of LNnT at 1.50 and 2.69 g/person/day, respectively. The summary of the estimated dietary intake of LNnT from use in infant formula and all proposed foods in the infant and toddler population, as described in GRN 547, is provided in Table 3.1.2.2-1 (U.S. FDA, 2015). Dietary intakes of LNnT presented in Table 3.1.3-1 below are fully substitutional to those described in GRN 659 and therefore no change in dietary intake is expected from the introduction of Inbiose's ingredient to the U.S. marketplace.

Table 3.1.2.2-1 Summary of the Estimated Daily Intake of LNnT from Infant Formulas and from all Proposed Food Uses in the U.S. Infant and Toddler Population Groups (2009-2010 NHANES Data)[a]

Population Group	Age Group (Years)	All-Person Consumption (g/day)		All-Users Consumption (g/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Infants	0 to 6 months	0.66	1.56	80.5	168	0.82	1.60
Infants	7 to 12 months	1.50	2.69	100	161	1.50	2.69
Toddlers	1 to 3	0.66	1.21	99.7	644	0.67	1.21

GRN = GRAS Notice; LNnT = lacto-*N*-neotetraose; NHANES = National Health and Nutrition Examination Survey.
[a] Table adapted from GRN 547 (U.S. FDA, 2015), full intake assessment reported in GRN 547 GRAS determination.

3.2 Estimated Intake of LNnT (General U.S. Population)

3.2.1 Methods

The estimated daily intake of LNnT in the infant and toddler population group was addressed in GRN 547 (Section IV.A, page 17). The estimated daily intake of LNnT from its use in conventional food and beverage products in the general U.S. population was addressed in GRN 659 (Section IV.A, page 27) (see Table 1.3-1). Proposed food uses include beverages and beverage bases up to 2.5 g/L, dairy product analogs up to 0.58 g/L or 2.67 g/kg, grain products and pastas up to 20 g/kg, milk (whole and skim) up to 0.58 g/L, milk products up to 2.5 g/L or 2.67 g/kg and processed fruits and fruit juices up to 0.58 g/L. These intended use levels are based on the levels of LNnT consumed by infants from human breast milk, as described in GRN 547. As this ingredient would serve as an alternative source of LNnT, additive increases in LNnT consumption are not expected to occur.

3.2.2 Intake Estimates for LNnT

As described in GRN 659, the estimated intake of LNnT as an ingredient in toddler formula and other food and beverage products has been estimated from dietary survey data. The intake of LNnT described in GRN 659 was estimated using food categories representative of each proposed food use chosen from the National Center for Health Statistics' 2011-2012 NHANES (CDC, 2015; USDA, 2014). Based on the proposed uses, more than 85% of the evaluated population groups consisted of eligible LNnT consumers, with toddlers established to represent the highest mean and 90th percentile consumer-only intakes of LNnT on an absolute basis, at 514 mg/person/day and 901 mg/person/day, respectively. The summary of the estimated dietary intake of LNnT in the U.S. population, as described in GRN 659, is provided in Table 3.2.2-1 (U.S. FDA, 2016). Dietary intakes of LNnT presented in Table 3.2.2-1 below are fully substitutional to those described in GRN 659 and therefore no change in dietary intake is expected from the introduction of Inbiose's ingredient to the U.S. marketplace.

Table 3.2.2-1 Summary of the Estimated Daily Intake of LNnT from All Proposed Food and Beverage Uses in the U.S. by Population Group (2011-2012 NHANES Data)[a]

Population Group	Age Group (Years)	All-Person Consumption (mg/day)		All-Users Consumption (mg/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Toddlers	1 to 3	510	901	99.3	561	514	901
Children	4 to10	344	648	99.1	1,161	347	648
Female Teenagers	11 to 18	222	529	90.0	513	247	546
Male Teenagers	11 to 18	288	627	90.3	476	319	632
Female Adults of Child Bearing Age	19 to 40	212	500	85.0	702	249	526
Female Adults	19 to 64	202	493	81.6	1,448	248	541
Male Adults	19 to 64	253	634	79.9	1,318	317	680
Elderly Adults	65 and up	214	513	88.2	818	243	550
Total Population	All Ages	259	609	85.2	6,595	304	646

GRN = GRAS Notice; LNnT = lacto-*N*-neotetraose; NHANES = National Health and Nutrition Examination Survey.
[a] Table adapted from GRN 659 (U.S. FDA, 2016), full intake assessment reported in GRN 547 GRAS determination.

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

6.1 Introduction

The first GRAS conclusion notified to the U.S. FDA for LNnT was submitted by Glycom in 2014 (GRN 547; U.S. FDA, 2015). A critical and comprehensive review of the publicly available data and information pertaining to the safety of LNnT for use as an ingredient in non-exempt infant formula, and various food and beverage products across multiple categories was presented in the Notice, and the published information pertinent to safety of LNnT presented by Glycom has served as the basis for subsequent GRAS conclusions for similar LNnT preparations (U.S. FDA, 2016, 2020f,g). These LNnT preparations are produced *via* chemical synthesis and microbial fermentation using genetically modified strains of *E. coli* K-12 DH1 or *E. coli* BL21 DE3. Despite differences in manufacturing process, these LNnT ingredients are all compositionally highly similar (see Table 2.3.1-1) and therefore safety data conducted with any of these ingredients are generally applicable to all ingredients. Within the previous GRAS Notices, data and information supporting the GRAS use of LNnT as an ingredient in infant formula and other foods have been critically reviewed by a number of qualified scientific experts, including the FDA, and are publicly available. Additionally, EFSA has issued multiple opinions supporting the safe use of LNnT as an ingredient in a variety of foods, including infant and follow-on formula (EFSA, 2015, 2020).

As reported in Section III Part B of GRN 919, concentrations of LNnT in human milk can range from 110 to 630 mg/L, varying depending on ethnicity, Secretor and Lewis-blood type, lactation period, and term *vs*. preterm birth. These LNnT levels in human breast milk have been measured in a wide range of previously-evaluated studies (Chaturvedi *et al.*, 1997, 2001; Thurl *et al.*, 1997, 2010; Coppa *et al.*, 1999; Nakhla *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Asakuma *et al.*, 2008; Leo *et al.*, 2010; Asakuma *et al.*, 2011; Gabrielli *et al.*, 2011; Galeotti *et al.*, 2012; Bao *et al.*, 2013; Austin *et al.*, 2016, 2019; Sprenger *et al.*, 2017; Williams *et al.*, 2017). As such, the use of LNnT as an ingredient in non-exempt term infant formula at levels up to 0.6 g/L is within the range that infants are exposed to following the ingestion of human milk.

Based on the equivalence of Inbiose's LNnT to other LNnT with GRAS status, publicly available data and information establishing the GRAS status of LNnT (U.S. FDA, 2015, 2016, 2020f,g) are therefore incorporated by reference to previous GRAS evaluations in the sections below. Since the most recent GRAS conclusion notified to the U.S. FDA was in 2020, an updated comprehensive search of the publicly available scientific literature was conducted to identify new information relevant to the safety of LNnT published through April 2021. The following databases were accessed: AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine, BIOSIS Toxicology, BIOSIS Previews, CAB ABSTRACTS, Embase, Foodline: SCIENCE, FSTA, MEDLINE, NTIS: National Technical Information Service, Toxicology Abstracts, and ToxFile. A summary of the historical basis for the GRAS determination of LNnT and any newly identified studies relevant to the safety of Inbiose's LNnT are provided below.

6.2 Absorption, Distribution, Metabolism, and Excretion

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

The metabolism of HMOs, including LNnT, has been previously described in detail (U.S. FDA, 2015, 2016, 2020f,g). Briefly, HMOs are resistant to enzymatic hydrolysis and are therefore not significantly digested in the upper gastrointestinal tract (Brand-Miller *et al.*, 1998; Engfer *et al.*, 2000; Rudloff and Kunz, 2012; EFSA, 2020). Once in the large intestine, LNnT is metabolized by the intestinal microbiota. The effects of HMOs on gastrointestinal bacterial growth are bacterial strain and HMO structure-dependent. Different growth patterns were observed for different bacteria strains when exposed to the same HMOs *in vitro* (Cheng *et al.*, 2021). In an *in vitro* study of various sialyllactoses treated with artificial gastric fluid, Gnoth *et al.* (2000) observed only minor structural changes in the HMOs and concluded that <5% of those ingested would be digested and subsequently absorbed. In breastfed infants, only minimal levels of ingested HMOs have been detected unchanged in the urine (*i.e.,* 1 to 2% of the total HMO fraction).

In an *in vivo* study, Vazquez *et al*. (2017) investigated the absorption of LNnT, after its oral administration in neonatal and adult Sprague-Dawley rats. In this experiment, pups ranging from 9 to 11 days of age (4 pups/sex/dose) received a soy formula with or without LNnT. LNnT was dosed *via* gavage at concentrations of 0.2 or 1 g/kg body weight. The toxicokinetic blood plasma profile of LNnT was determined after gavage at 30, 60, 90, 120, 180, and 240 minutes post-dose. Twelve pups (6 pups/sex) were used to obtain basal levels. In another experiment contained within this study, conducted in adult rats, females (8/dose) were administered the same dose levels of LNnT. LNnT was dissolved in water (vehicle) and administered by gavage after 12 hours of fasting. Blood samples were collected at 30, 60, 90, 120, 180, 240, and 300 minutes post-dose. In both experiments, LNnT was not detected in the blood serum of any animals at baseline. LNnT was measurable in the serum samples of animals at both dose levels 30 minutes post-dose. However, slightly different toxicokinetic profiles were noted after exposure to low and high dose of LNnT in adult rats. At the lowest dose, the peak was reached after 60 minutes, and the serum LNnT level began to decrease slowly in plasma at the 90 minute time point . At the highest dose of LNnT, a first peak appeared at 30 minutes, followed by a 90-minute plateau phase, after which the level of serum LNnT reached the maximum level (180 minutes) and before beginning to decline (Vazquez *et al*. (2017). In contrast to blood serum, LNnT was detected in urine samples at baseline in all sampling times.

6.3 Toxicological Studies

6.3.1 Toxicology Studies Conducted with Inbiose's LNnT

Toxicology studies characterizing the genotoxicity and subchronic toxicity of LNnT in neonatal rats are presented as conducted information on the safety of the ingredient. Findings from these studies are consistent with observations previously reported in the published literature and described in GRN 659 and 919: LNnT is not genotoxic and does not pose a toxicological safety concern.

6.3.1.1 Genotoxicity

Bacterial Reverse Mutation (Ames) Test (OECD Test Guideline 471)

The potential mutagenicity of Inbiose's LNnT was evaluated in a bacterial reverse mutation assay conducted in accordance with OECD Test Guideline 471 (OECD, 1997a) (Tóth-Gönczöl, 2021 [unpublished]). The study consisted of a Preliminary Range Finding Test (Plate Incorporation Method), Assay 1 (Plate Incorporation Method), and Assay 2 (Plate Incorporation Method without metabolic activation and Pre-Incubation Method with metabolic activation). Due to observed contaminations on the plates conducted with *Salmonella* Typhimurium TA100 strain with and without metabolic activation in Assay 1, the results could not be properly evaluated. Therefore, the Assay 1 portion of this study conducted with this strain was

declared invalid and was repeated in an additional experiment, labelled Assay 3, and was conducted under the same experimental conditions as Assay 1.

For the range finding test, *Salmonella* Typhimurium TA98 and TA100 tester strains and *E. coli* strain WP2 *uvrA* were exposed to LNnT at 10, 31.6, 100, 316, 1,000, 2,500, or 5,000 µg/plate in the absence and presence of metabolic activation. For the plate incorporation and pre-incubation method *S.* Typhimurium strains TA98 (pKM101), TA100 (pKM101), TA1535, and TA1537 and *E. coli* strain WP2 *uvrA* were exposed to LNnT at concentrations of 15.81, 50, 158.1, 500, 1,581, or 5,000 µg/plate (the OECD Test Guideline 471 maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix prepared from the livers of phenobarbital/β-naphthoflavone-induced rats). Water for injection served as the vehicle for LNnT and as the negative control. Positive controls were also included in the presence (2-aminoanthracene) and absence (9-aminoacridine, sodium azide, 4-nitro-*o*-phenylenediamine and methyl methanesulfonate) of metabolic activation. The full list of controls is provided below in Table 6.3.1.1-1.

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

Without Metabolic Activation (-S9 mix)					
Test Strains	**TA 98**	**TA 100**	**TA 1535**	**TA 1357**	**WP2 uvrA**
Positive control -S9 mix/+S9 mix	NPD 4 µg/plate/ 2AA 2 µg/plate	NaN_3 2 µg/plate/ 2AA 2 µg/plate	NaN_3 2 µg/plate/ 2AA 2 µg/plate	9AA 50 µg/plate/ 2AA 2 µg/plate	MMS 2 µL/plate/ 2AA 50 µg/plate
With Metabolic Activation (+S9 mix)					
Test Strains	**TA 98**	**TA 100**	**TA 1535**	**TA 1357**	**WP2 uvrA**
Positive control	NPD 4 µg/plate/ 2AA 2 µg/plate	NaN_3 2 µg/plate/ 2AA 2 µg/plate	NaN_3 2 µg/plate/ 2AA 2 µg/plate	9AA 50 µg/plate/ 2AA 2 µg/plate	MMS 2 µL/plate/ 2AA 50 µg/plate

2AA = 2-aminoanthracene; 9AA = 9-aminoacridine; MMS = methyl-methanesulfonate; NaN_3 = sodium azide; NPD = 4-nitro-1,2-phenylene-diamine; S9 = metabolic activation.

There was no evidence of mutagenicity in either test, in the absence or presence of metabolic activation. When compared to the vehicle control, higher numbers of revertant colonies were sporadically observed in the main test results; however, a dose-dependent response could not be established, and they were below the biologically relevant threshold value. Thus, they were considered as reflecting the biological variability of the test. Some sporadic lower revertant counts were also observed in the main test at select non-cytotoxic concentrations; however, no background inhibition was recorded and the mean numbers of revertant colonies were in the historical control range in all cases. Thus, they were considered as biological variability of the test system. No precipitation, growth inhibition, or cytotoxic effects related to LNnT were observed in the main assays in all of the examined bacterial strains.

The positive controls showed a distinct increase of induced revertant colonies in each strain with and without metabolic activation. The mean values of revertant colonies of the negative (vehicle/solvent) control plates were within the historical control range. The viability of the bacterial cells was checked by a plating experiment in each test and the study was considered to be valid. Under the experimental conditions applied in this study, LNnT did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. The results of Inbiose's bacterial reverse mutation assay are provided below in Table 6.3.1.1-2.

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

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

Concentration (µg/plate)	Revertant Colonies per Plate (Mean ± SD)									
	Without Metabolic Activation (-S9 mix)					With Metabolic Activation (+S9 mix)				
	Salmonella Typhimurium				*Escherichia coli*	*Salmonella* Typhimurium				*Escherichia coli*
	TA98	TA100*	TA1535	TA1537	WP2unA	TA98	TA100*	TA1535	TA1537	WP2unA
Plate Incorporation Method – Assay 1/3*(Mean ± SD)										
Water for injection	17.7± 0.58	85.0±2.00	12.7±0.58	9.3±2.52	50.0±1.00	19.0±1.00	97.7±4.16	14.0±2.00	12.7±1.53	50.0±1.00
5,000	15.3±1.15	88.3±7.57	12.3±2.08	8.3±1.15	48.7±0.58	18.7±1.53	94.0±2.00	11.7±1.53	8.7±1.15	52.0±3.46
1,581	16.7±0.58	83.3±3.06	12.0±3.00	7.7±0.58	50.7±1.15	18.3±1.53	94.3±17.16	10.7±3.06	7.7±0.58	53.7±1.53
500	17.7±0.58	86.7±3.21	12.3±2.08	9.3±1.15	49.3±1.53	19.0±1.00	98.0±12.53	9.0±2.00	9.3±1.15	49.7±4.04
158.1	16.7±1.15	84.7±8.50	11.0±1.00	8.3±1.53	49.0±1.00	18.0±1.00	105.0±6.56	11.3±2.31	8.3±1.53	49.0±3.00
50	15.7±0.58	81.0±5.57	12.7±2.31	9.0±1.00	48.7±2.52	17.7±0.58	108.0±5.20	12.7±2.52	9.0±1.00	51.3±2.08
15.81	16.0±0.00	82.0±6.00	15.3±1.53	9.0±1.73	49.7±2.08	17.7±0.58	105.7±8.14	10.7±1.15	9.0±1.73	51.3±3.06
Positive control[a]	425.3±16.65	1,088.0±38.16	1,054.7±126.26	490.7±32.33	1,093.3±28.10	2,416.0±42.33	2,466.7±16.17	222.7±14.05	212.0±14.42	252.0±8.00
Plate Incorporation Method (-S9- mix), Pre-Incubation Method (+S9-mix) – Assay 2 (Mean ± SD)										
Water for injection	17.0±1.00	101.7±7.64	14.7±0.58	10.7±2.31	50.3±5.51	22.0±1.00	106.3±6.35	14.0±0.00	12.7±1.15	72.7±5.51
5,000	17.3±0.58	89.0±7.55	13.3±0.58	6.3±3.51	51.3±19.01	21.0±1.00	112.7±1.15	12.3±0.58	8.7±2.08	69.0±8.54
1,581	17.3±1.15	98.3±1.15	13.7±1.15	11.0±1.00	51.0±2.00	22.3±0.58	101.7±10.69	14.0±1.00	10.7±4.16	77.0±6.24
500	18.7±1.15	94.0±5.29	13.3±0.58	9.0±2.00	59.0±7.00	19.0±1.00	102.3±3.51	13.3±1.15	11.3±1.15	81.0±6.93
158.1	18.7±1.15	93.7±9.29	14.0±0.00	9.3±2.31	60.7±7.37	21.7±3.06	106.3±6.35	12.7±1.15	11.0±1.73	77.7±8.08
50	19.0±1.00	95.7±9.07	14.0±1.00	9.3±2.31	62.3±3.51	18.0±1.00	103.7±5.86	12.3±0.58	13.3±1.15	89.3±6.11
15.81	16.3±0.58	91.7±8.50	13.3±0.58	9.3±2.08	55.7±5.69	21.0±2.65	108.0±5.29	12.7±0.58	10.3±0.58	78.3±11.06
Positive control[a]	404.0±14.42	1,054.7±18.04	1,168.0±49.96	406.7±18.04	1,170.7±24.44	2,421.3±32.33	2,482.7±12.22	212.0±10.00	213.3±10.07	242.0±7.21

S9 = metabolic activation; SD = standard deviation.

* The performed Assay I in case of *Salmonella* Typhimurium TA100 strain with and without metabolic activation could not be properly evaluated due to the observed contamination on the plates. Therefore, the performed Assay 1 in case of this strain was declared invalid and was repeated in an additional experiment (Assay 3) with and without metabolic activation with the same experimental conditions as in Assay 1.

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

6.3.1.2 Acute Oral Toxicity Test (OECD Test Guideline 425)

The median lethal dose (LD_{50}) of LNnT was assessed in a single dose acute toxicity study conducted in accordance with OECD Test Guideline 425 (OECD, 2008a) (Orosz, 2021 [unpublished]). Three female Crl:WI Wistar rats were administered a single dose of 5,000 mg LNnT/kg body weight dissolved in distilled water for injection *via* gavage, followed by a 14-day observation period. No mortality or test item-related effects were observed; hence, the LD_{50} was concluded to be greater than 5,000 mg LNnT/kg body weight (the OECD Test Guideline 425 maximum recommended concentration).

6.3.1.3 Repeat Dose Toxicity Study (Dose Range Finding Study)

A non-Good Laboratory Practice (GLP) 21-day repeat dose toxicity study was conducted in juvenile Sprague-Dawley (SD) rats to evaluate the short-term toxicity of LNnT and select a maximum dose for the subsequent 90-day subchronic toxicity study (Bentz, 2021 [unpublished]).

Groups of 8 male and female juvenile SD rats were administered 0 (sterile water for injection, vehicle) 3,000, 4,000, or 5,000 mg LNnT/kg/day by gavage from Post-natal Day (PND) 7 to 27 at 10 mL/kg/day under the constant dose-volume of 10 mL/kg/day. A satellite group of SD rats (4/sex) were also allocated to the 5,000 mg/kg body weight (bw)/day group for the development and further validation of bioanalytical method of detection of LNnT in plasma and urine. All animals were observed daily for clinical signs and any changes in body weight and food consumption before being subjected to necropsy. Hematology and blood chemistry parameters were measured at the end of the treatment period.

On PND 28 (after at least 14 hours fasting), the animals were euthanized and a complete macroscopic *post-mortem* examination of the principal thoracic and abdominal organs was performed. Selected organs were weighed and a full list of organs were preserved. Satellite rats received LNnT by oral gavage daily until PND 22. Blood samples were collected on PND 22 at designated time-points, (*i.e.*, 0, 1, 4, and 6 hours after dosing). Urine samples were collected after at least 24 hours had passed since dose administration. Satellite animals were euthanized after urine collection and were not necropsied.

There were no unscheduled deaths attributed to treatment with LNnT during the study. At 5,000 mg/kg bw/day, a decrease in terminal body weight was observed in both sexes: 12% and 10% in males and females, respectively. A minimal decrease in body weight (approximately 15%) was also observed in both sexes just after weaning (PND 21 to 24). A similar trend was observed in males of the 4,000 mg/kg bw/day group. A decrease in the absolute and relative mean liver weights in both sexes and a decrease in the absolute mean kidney weights at 5,000 mg/kg bw/day was considered to be related to this decrease in body weight and food consumption (Levin *et al.*, 1993). There were no macroscopic observations related to the test item administration. A decrease in the relative weight of brains of both sexes at 5,000 mg/kg bw/day was linked to the statistically significant changes in final body weights. Slight decreases in mean absolute (statistically significant) and relative-to-body weights were observed for the spleen in females at 4,000 and 5,000 mg/kg bw/day and for the thymus in males at 5,000 mg/kg bw/day. However, changes in relative weights were not statistically significant. Moderate increases, sometimes statistically significant, were observed in the absolute and relative-to-body mean adrenal and thyroid glands weights in both sexes at 3,000 and 4,000 mg/kg bw/day; however, this was not dose-related, and thus, these findings were considered to be of equivocal relationship with the test item administration. No macroscopic observations related to the test item administration were noted.

When compared to the control, a decreased mean leucocyte and lymphocyte counts were noted in both sexes at 5,000 mg/kg bw/day and in males of the 4,000 mg/kg bw/day group. This trend was considered

likely to be secondary stress linked to a reduction of body weight and food consumption. The observed statistically significantly decrease in mean cell hemoglobin in females at 4,000 mg/kg bw/day, was not considered to be test item-related. This change was of minimal magnitude, lacked dose relationship, did not correlate with any effect on red blood cells, and was not consistent between both sexes. The slight, yet statistically significant decrease in mean total protein and albumin levels observed only in females of the high-dose group were of minimal magnitude. The individual values were within the range of reference data. The mean albumin/globulin ratio was comparable between treatment and control groups.

Mean cholesterol and triglyceride levels were decreased in both sexes at all dose levels *versus* controls, reaching statistical significance; however, these changes lacked a clear dose-relationship. The individual values were within the range of reference or concurrent control data, except for 1 male and 1 female at 3,000 mg/kg bw/day for cholesterol. Some animals (1 male in each group and 1 high-dose female) showed an abnormally high potassium value (between 6.52 and 8.04 mmol/L) not necessarily compatible with life; these data were considered to be abnormal and not representative of the reality. This type of event can be observed when blood sampling is performed at necropsy in rodents.

The other statistically significant differences when compared with controls were considered of no relevance. The changes were of low magnitude, lacked dose-relationship, were not observed at the highest dose level, were of no clinical significance, did not correlate with other biochemistry mean data and/or were not consistent between both sexes. The statistically significant observations from this study are provided below in Table 6.3.1.3-1.

Based on the experimental conditions of this study, the repeated oral administration (*via* gavage) of LNnT at 3,000, 4,000, or 5,000 mg/kg bw/day in juvenile rats for 21 days did not elicit any signs of adverse toxicity; therefore, 5,000 mg/kg bw/day, the highest tested dose of LNnT in this study, was considered to be an acceptable high dose-level in the 90-day juvenile rat study.

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

Parameters	Exposure	Sex	Dose Group (LNnT mg/kg bw/day)			
			0	3,000	4,000	5,000
Body weight/Mean Body Weight Change (Mean values ± SD)						
Body weight (g) (1)	Day 11	M	46±2.6	50*±2.2	48±4.2	43±3.8
Body weight (g) (1)	Day 15	M	61±3.7	64±2.1	61±5.3	55*±4.8
Body weight (g) (1)	Day 18	M	78±3.8	80±3.5	75±6.3	69**±3.9
Body weight (g) (1)	Day 18	F	74±6.8	76±4.7	75±4.5	68*±3.2
Body weight (g) (1)	Day 21	M	97±3.9	99±4.1	94±7.3	85**±3.9
Body weight (g) (1)	Day 21	F	91±6.9	91±5.5	91±5.5	83*±4.3
Body weight change (g) (3) SD (K)	Day 8/11	F	8±1.1	10##±0.5	9±0.4	8±0.8
Body weight change (g) (1)	Day 11/15	M	16±2.1	14±1.2	14±1.6	12**±1.6
Body weight change (g) (3)SD (K)	Day 15/18	M	17±1.4	17±2.4	14#±2.0	13#±1.8
Body weight change (g) (1)	Day 15/18	F	14±1.5	14±2.2	12*±2.9	11**±1.9
Body weight change (g) (1)	Day 1/21	M	79±3.2	80±3.4	76±6.0	68**±2.8
Body weight change (g) (1)	Day 1/21	F	73±5.7	74±5.4	73±4.7	66*±3.8
Final Body Weight/Organ Weights (Mean values ± SD)						
Final mean weight (g) (1)	Day 22	M	91.25±3.68	93.50±3.14	88.51±6.22	79.85**±3.95
Final mean weight (g) (1)	Day 22	F	85.89±6.46	86.05±4.64	86.18±4.49	77.43**±4.88
Adrenal glands (g) (1) Mean weight	Day 22	M	0.02750±0.004	0.03325*±0.005	0.03463 **±0.003	0.02363±0.003
Adrenal glands (g) (1) Mean % body	Day 22	M	0.03013±0.004	0.03554 *±0.006	0.03923**±0.003	0.02968±0.004
Adrenal glands (g) (3) Mean % body	Day 22	F	0.03005±0.002	0.03679#±0.006	0.03661#±0.006	0.03430±0.008
Brain (g) (1) Mean % body	Day 22	M	1.78±0.061	1.78±0.085	1.83±0.117	1.98**±0.096
Brain (g) (1) Mean % body	Day 22	F	1.83±0.118	1.86±0.098	1.91±0.076	2.02**±0.127
Kidneys (g) (3) Mean weight	Day 22	M	0.97025±0.032	1.02±0.042	0.94938±0.070	0.85400#±0.023
Liver (g) (3) Mean weight	Day 22	M	3.16±0.088	3.25±0.373	3.22±0.255	2.66##±0.122
Liver (g) (3) Mean weight	Day 22	F	3.01±0.216	2.95±0.230	2.87±0.143	2.61##±0.160

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

Parameters	Exposure	Sex	Dose Group (LNnT mg/kg bw/day)			
			0	3,000	4,000	5,000
Spleen (g) (1) Mean weight	Day 22	F	0.29725±0.043	0.30838±0.025	0.25888*±0.025	0.24213**±0.025
Thyroid glands (g) (3) Mean weight	Day 22	F	0.00813±0.001	0.01075##±0.001	0.01025#±0.001	0.00913±0.002
Hematology (Mean values ± SD)						
MCH (pg) (1)	Day 22	F	22.1±0.38	21.5±1.00	20.9 **±0.64	21.3±0.51
Blood Biochemistry (Mean values ± SD)						
GLUC (mmol/L) (1) SD (L)	Day 22	M	6.52±0.633	7.50±1.340	9.28 **±2.565	7.97±1.208
CREAT (µmol/L) (1)	Day 22	M	19.69±2.074	16.28 **±1.708	16.84 *±1.138	15.14**±2.488
CHOL (mmol/L) (1)	Day 22	M	2.48±0.303	2.03 **±0.182	2.04**±0.265	2.13 *±0.160
CHOL (mmol/L) (1)	Day 22	F	2.44±0.213	1.86 **±0.209	1.92 **±0.306	1.90 **±0.207
TRIG (mmol/L) (1)	Day 22	M	0.78±0.187	0.36 **±0.124	0.37 **±0.103	0.41**±0.113
TRIG (mmol/L) (1)	Day 22	F	0.48±0.176	0.35±0.114	0.33±0.146	0.29 *±0.056
ALP (U/L) (1)	Day 22	M	806±89.0	657 **±76.2	681 **±56.6	708 *±77.6
K+ (mmol/L) (3) n (B)	Day 22	F	3.65±0.502	4.28±1.098	3.67±0.189	4.57#±1.090
Cl- (mmol/L) (1)	Day 22	F	108.4±1.19	106.5 **±0.79	106.2 **±1.23	106.2 **±1.10
PROT (g/L) (1)	Day 22	F	46.9±0.87	45.5±1.51	45.8±1.34	44.5 **±0.96
ALB (g/L) (3) SD (K)	Day 22	F	34±1.2	33±0.9	33±1.1	32##±0.7

ALB = albumin; bw = body weight; CHO = total cholesterol; Cl- = chloride; CREAT = creatinine; F = female; GLUC = glucose; K+ = potassium; LNnT = lacto-*N*-neotetraose; M = male; MCH = mean cell hemoglobin; PROT = total proteins; SD = standard deviation; TRIG = triglycerides.

* P<0.05, ** P<0.01 (1) DUNNETT TEST

# P<0.05, ## P<0.01 (3) DUNN TEST

(B) BARTLETT TEST P<0.01

(K) KOLMOGOROV-LILLIEFORS TEST P<0.01

(L) LOGARITHMIC TRANSFORMATION Assigned control group(s): 1.

6.3.1.4 90-day Subchronic Toxicity Study (OECD Test Guideline 408) (OECD, 1998)

Currently, a 90-day study is being conducted to evaluate the potential toxic effects of Inbiose's LNnT on the development of juvenile rats, following daily oral administration, from PND 7 to at least PND 97 (Verchère-Beau, 2021 [unpublished draft report]). This study is ongoing, and a draft report is not available at the time of submitting this notice. The results of this study will be provided as supplemental information to the notice once available.

6.3.1.5 Summary of Studies Conducted with Inbiose's LNnT

Pertinent studies conducted with Inbiose's LNnT are summarized in Table 6.3.1.5-1, below. The results of these studies indicate no evidence of toxicity related to the administration of LNnT.

Table 6.3.1.5-1 Summary of Toxicological Studies to Support the Safety of Inbiose's LNnT

Type of Study	Species or Cell Type	Length of Study	Dose and Route of Administration	Result	Reference
Studies Conducted with Inbiose´s LNnT					
Bacterial reverse mutation test (OECD TG 471)	*S.* Typhimurium strains TA98 (pKM101), TA100 (pKM101), TA1535, and TA1537 and *E. coli* strain WP2 uvrA	Plate incorporation assay and pre-incubation method	0 (water for injection, vehicle); up to 5,000 µg/plate (±S9 mix)	LNnT is non-mutagenic under the conditions of this test	Tóth-Gönczöl, [unpublished] 2021 final report
Single dose acute toxicity study up and down procedure (OECD TG 425)	Three female Crl:WI Wistar rats	Single dose followed by a 14-day observation period	5,000 mg/kg bw LNnT dissolved in water for injection – the highest recommended dose	LD_{50} of LNnT was found to be greater than 5,000 mg/kg bw	Orosz, [unpublished] 2021 final report
Preliminary toxicity study by oral route (gavage) in Juvenile rats	Group of 8 male and 8 female juvenile SD rats	21 days from PND 7	0 (water for injection, vehicle) 3,000, 4,000 or 5,000 mg/kg bw/day of LNnT, by gavage	5,000 mg/kg bw/day was selected as the appropriate highest dose for the main 90-day study	Bentz, [unpublished] 2021 final report
90-day toxicity study by oral route (gavage) in Juvenile rats followed by a 4-week Treatment-Free Period (OECD TG 408)	Group of 10 male and 10 female juvenile SD rats	90 days from PND 7	0 (water for injection, vehicle) 3,000, 4,000 or 5,000 mg/kg bw/day of LNnT, by gavage	In progress	Verchère-Beau [unpublished] 2021 draft report

bw = body weight; *E. coli* = *Escherichia coli*; LD_{50} = median lethal dose; LNnT = lacto-*N*-neotetraose; OECD = Organisation for Economic Co-operation and Development; PND= Post-natal Day; S9 = metabolic activation mix; *S.* Typhimurium = *Salmonella* Typhimurium; SD = Sprague-Dawley; TG = Test Guideline.

6.3.2 Pre-clinical Studies Conducted with Other LNnT Ingredients

Pivotal safety data and information has been discussed previously and is hereby incorporated by reference to Section IV.B.5 of GRN 547 and Section IV.E of GRN 659 (U.S. FDA, 2015, 2016). Analytical data of Inbiose's LNnT product establishes the ingredient as chemically identical to its LNnT counterpart in human breast milk (see Section 2.1.1). Based on analytical data presented demonstrating that LNnT produced by Inbiose is of equal or greater purity to LNnT preparations that have previously been determined to be GRAS, studies characterizing the toxicity and safety of LNnT in animal models are considered relevant to the safety assessment of Inbiose's ingredient. No evidence of toxicity related to the administration of LNnT has been reported in any previous LNnT GRN submission (U.S. FDA, 2015, 2016, 2020f,g). Additionally, there were no new data identified evaluating the potential toxicological or genotoxic effects of the ingredient since the previous LNnT GRAS determination was prepared. The toxicological studies in GRN 547 and 659 are briefly summarized below in Table 6.3.2-1, and the summaries are presented in the following sections.

Table 6.3.2-1 Summary of Toxicological Studies to Support the Safety of Inbiose's LNnT

Type of Study	Species or Cell Type	Length of Study	LNnT Dose and Route of administration	Result	Reference
Studies Conducted with Glycom's LNnT (GRN 547)					
Bacterial reverse mutation test	*S.* Typhimurium strains TA98, TA100, TA1535, TA1537, and TA102	Plate incorporation assay and pre-incubation assay	Up to 5,000 µg/plate (±S9)	LNnT is non-mutagenic at concentrations up to 5,000 µg/plate	Coulet *et al.* (2013)
In vitro mammalian cell gene mutation test	L5178Y tk+/- mouse lymphoma cells	-S9 = 24 hours ±S9 = 4 hours	-S9 = 1.4 to 4,250 µg/mL ±S9 = 418 to 4,250 µg/mL	LNnT is non-mutagenic in L5178Y tk+/-mouse lymphoma cells	
14-day oral toxicity study	Groups of 5 male and 5 female Wistar [Crl:WI(Han)] pups	14 days	0 (water vehicle control), 1,000, 2,500, or 5,000 mg/kg bw/day LNnT, by gavage 5,000 mg OF/kg bw/day = reference control	5,000 mg/kg bw/day LNnT is suitable for longer term studies	
28-day oral toxicity study	Groups of 5 male and 5 female Wistar [Crl:WI(Han)] pups	28 days	0 (water vehicle control), 1,000, 2,500, or 5,000 mg/kg bw/day LNnT, by gavage 5,000 mg OF/kg bw/day = reference control	NOAEL is 5,000 mg/kg bw/day of LNnT	

Table 6.3.2-1 Summary of Toxicological Studies to Support the Safety of Inbiose's LNnT

Type of Study	Species or Cell Type	Length of Study	LNnT Dose and Route of administration	Result	Reference
90-day oral toxicity study	Groups of 15 male and 15 female Wistar [Crl:WI(Han)] pups	90 days	0 (water vehicle control), 1,000, 2,500, or 5,000 mg/kg bw/day LNnT, by gavage 5,000 mg OF/kg bw/day = reference control	NOAEL is 5,000 mg/kg bw/day of LNnT	
Other Studies Identified in GRN 547					
28-day oral toxicity study	12 litters (5/sex/litter) of Cri:CD®BR rat pups (15 days old)	28 days	0 (control), 10, 200, or 400 mg/kg bw/day of the LNnT test article (purity not reported) *via* gavage	No significant differences in any of the evaluated parameters	Prieto (2005)
4-month oral toxicity study	31-to 37-day-old rats (sex, strain, and number NR)	4 months	1 or 5% LNnT (equivalent to approximately 1,000 and 5,000 mg/kg bw/day, respectively	No test article-related adverse effects or macroscopic and microscopic changes were observed following LNnT administration	
Studies Conducted with Glycom's LNnT (GRN 659)					
Bacterial reverse mutation assay	*S.* Typhimurium strains TA98, TA100, TA102, TA1535, and TA1537 and *E. coli* WP2uvrA	Plate incorporation assay and pre-incubation assay	Up to 5,000 µg/plate (±S9)	LNnT is non-mutagenic at concentrations up to 5,000 µg/plate	Verspeek-Rip (2016)
In vitro micronucleus assay	Human lymphocytes	±S9 = 3 hours -S9 = 24 hours	±S9 = 512, 1,600, or 2,000 µg/mL -S9 = 512, 1,600, or 2,000 µg/mL	Under the conditions tested, LNnT was determined to be non-clastogenic and non-aneugenic in human lymphocytes	Verbaan (2016)
90-day oral toxicity study	Groups of 10 male and 10 female, 7-day-old weanling Wistar [Crl:WI(Han)] rats	90 days	0 (water vehicle control), 1,000, 2,500, or 5,000 mg/kg bw/day LNnT, by gavage 5,000 mg FOS/kg bw/day = reference control	NOAEL is 5,000 mg/kg bw/day of LNnT	Penard (2016)

bw = body weight; *E. coli* = *Escherichia coli*; FOS = fructo-oligosaccharide; GRN = GRAS Notice; LNnT = Lacto-*N*-neotetraose; NOAEL = no-observed-adverse-effect level; NR = not reported; OF = oligofructose; *S.* Typhimurium = *Salmonella* Typhimurium.

6.3.2.1 Summaries of Genotoxicity Studies Conducted with Other LNnT Ingredients

Genotoxicity studies of other LNnT ingredients were reported in detail in previous GRAS Notices (GRN 547, 659, 895, and 919). Pivotal genotoxicity studies are summarized below.

LNnT produced by chemical synthesis was tested in the reverse mutation assay (OECD Test Guideline 471) for its ability to induce reverse mutations at selected loci of *S.* Typhimurium tester strains TA98, TA100, TA1535, TA1537, and TA102 in the presence and absence of metabolic activation using doses up to 5,000 µg per plate (Coulet *et al*., 2013; GRN 547). The negative control (water) and the positive controls depends on the bacterial strain (in the absence of S9-mix: 2-nitrofluorene (TA98), sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), or t-butyl hydroperoxide (TA102); in the presence of S9-mix, 2-aminoathracene) were tested in parallel. No cytotoxicity or precipitation was observed for any of the strains treated with LNnT in the presence or absence of S9. The positive controls induced the number of revertant colonies when compared to the negative control. In contrast, no mutagenic activity on the growth of the bacterial strains were reported after treatment with LNnT; therefore, LNnT was concluded to be non-mutagenic at concentrations up to 5,000 µg/plate.

In another reverse mutation assay conducted with LNnT (product of microbial fermentation), conducted in accordance with OECD Test Guideline 471, LNnT was concluded to be non-mutagenic when tested at concentrations of up to 5,000 µg/plate both in the presence and absence of metabolic activation (Verspeek-Rip, 2016 [unpublished]; GRN 659),

The clastogenic and aneugenic properties of LNnT, produced *via* chemical synthesis and microbial fermentation, were investigated in 2 separate *in vitro* assays. The chemically synthesized LNnT product was examined for its potential to induce gene mutations at the (TK)-locus of cultured mouse lymphoma L5178Y cells in both absence and presence of metabolic activation (S9-mix) for a short period of 4 h and longer treatment of 24 h without metabolic activation. This assay was performed according to OECD TG 476 (OECD, 1997b). LNnT was dissolved in water (vehicle) at the maximum feasible concertation of 4,250 µg/mL. Methyl methanesulfonate (MMS) and cyclophosphamide (CP) were used as positive controls for experiments without and with S9-mix, respectively. A test item was considered positive if a dose-dependent, statistically significant, mutant frequency increase is observed, with a statistically significant increase in average mutant frequency of at least 2-fold the negative control values. No significant increases in the mutagenicity or genotoxicity endpoints were observed (Coulet *et al*., 2013).

In the second assay, the genotoxicity of the crystallized LNnT form produced by microbial fermentation was investigated in an *in vitro* micronucleus assay conducted with cultured peripheral human lymphocytes (Verbaan, 2016, [unpublished], GRN 659). In both short- and long-term experiments, LNnT exposure at concentrations of 512, 1,600, or 2,000 µg/mL did not produce any statistically or biologically significant increases in the frequency of mono- or bi-nucleated cells with micronuclei. LNnT was concluded to be not genotoxic in human lymphocytes under the conditions of this assay.

6.3.2.2 Summaries of Pivotal Repeat Dose Toxicity Studies Conducted with Other LNnT Products

Three pivotal repeat dose toxicity studies of chemically synthesized LNnT were described by Coulet *et al*. (2013).

A tolerability 14-day dose-range finding (DRF) non-GLP pilot study conducted in the juvenile rats (5/sex/group) from PND 7 until PND 20 (weaning) was performed to define the dose levels for the subsequent longer term toxicity studies. The juvenile rats received LNnT by gavage at the dose levels of 1,000, 2,500 or 5,000 mg/kg bw/day for 14 days. The oral gavage LNnT treatment did not induce any test item-related deaths or effects. Therefore, the highest dose of 5,000 mg/kg bw/day LNnT was selected for further sub-acute 28-day and sub-chronic 90-day studies.

The 28-day study was conducted according to OECD Test Guideline 407 (OECD, 2008b). Juvenile rats (10/sex/group) received LNnT by oral gavage at 0 (water, vehicle), 1,000, 2,500 or 5,000 mg/kg bw/day from PND 7 for 28 days. A fifth group (Group V) of rats received the comparative control fructooligosaccharides (FOS) at 5,000 mg/kg bw/day for the same period of time.

The 90-day study was performed according to OECD Test Guideline 408 (OECD, 1998). Juvenile rats (10 animals/sex/group) received LNnT by gavage at 0 (water, vehicle), 1,000, 2,500, or 5,000 mg/kg bw/day for 90 consecutive days. A fifth group of rats received the comparative control FOS at 5,000 mg/kg bw/day for the same period of time. No statistically significant differences in mean body weights were observed in the LNnT exposed rats when compared to the control vehicle group in 28--and 90-day studies. Soft, liquid, colored (yellow) feces and erythema on the urogenital area were noted in animals treated with FOS from day 1 of the study until weaning (Study Days 13 to 14). Liquid feces were also observed in the LNnT high dose treated rats before weaning, but much less frequently (only on 2 occasions) and in few animals only (generally 1 to 4 daily). No other toxicologically relevant, test item-related changes in clinical observations, feed and water intake, biochemistry, hematology, urinalysis, organ weights, macroscopic or microscopic findings, mortality or morbidity were observed in LNnT-treated juvenile rats, were noted. Based on the results of the repeat 90-day study, the no-observed-adverse-effect level (NOAEL) was determined to be a least 5,000 mg/kg bw/day, the highest dose tested.

Another 90-day sub-chronic oral toxicity study (OECD Test Guideline 408, 1998) was conducted with crystallized form of LNnT product manufactured by microbial fermentation (Penard *et al.*, 2016 [unpublished]; GRN 659). The study followed the same design reported previously by Coulet *et al*. (2013). Briefly, the juvenile rats received LNnT by oral gavage at 0 (water, vehicle), 1,000, 2,500, or 5,000 mg/kg bw/day or FOS as a reference test item at concentration of 5,000 mg/kg bw/day for 90 or 91 days. An additional group of animals was used for a 28-day recovery period following 13-week of LNnT exposure. There were no test item-related mortalities during the study. The isolated occurrences of hypersalivation reported in 1 high LNnT-dose male and 3 high LNnT-dose females were considered to be a non-adverse. No other clinical signs were observed. No test item-related findings were reported in measured repro- and developmental or neurobehavioral parameters at any dose level. No significant effects in body weight, body weight gain, or food consumption were reported.

The statistically significant findings in hematology parameters were considered unrelated to LNnT administration as they were of minimal in magnitude and not dose dependent. No test item-related effects in serum biochemistry parameters were noted and any statistically significant differences in serum clinical chemistry parameters were ether observed in only 1 sex, and/or were within historic control values. The statistically significant increase in urine volume in high-dose animals and a statistically significant decrease in specific gravity were reported in high-dose animals compared with controls, were considered to be

incidental and of no toxicological relevance due to the lack of dose-response or histopathological changes in the kidney. No treatment-related differences in organ weights, macroscopic observations, or histological observations were reported between rats receiving LNnT and the control and reference groups. Based on the results, the NOAEL for fermentation-produced LNnT was determined to be 5,000 mg/kg bw/day, the highest dose tested.

Prieto (2005) has reported two 28-day oral gavage and 4-month dietary studies in neonatal and juvenile rats, respectively. LNnT product manufactured by yeast fermentation was used as a test item. In the 28-day study, 15-day-old rat pups (5/sex/litter) were administered control or 10, 200, or 400 mg LNnT/kg body weight/day *via* gavage. The evaluated parameters included urinalysis, hematology, fecal analysis, and gross pathology. It was concluded by author that no significant differences in measured parameters were reported between pups exposed up to 400 mg/kg bw/day when compared to the control pups. In the 4-month study, 31- to 37-day-old rats were fed diets containing 1 or 5% of LNnT for 4 months. There were no macroscopic or microscopic changes that appeared to be related to treatment except some unremarkable (unspecified by author) observations in the group fed with 5% LNnT.

6.4 Human Studies

Safety data and information provided from studies of the addition of LNnT to infant formula have been discussed previously and is hereby incorporated by reference to Section IV.B.6 of GRN 547, Section IV.F of GRN 659, and Section VI Part E of GRN 919 (U.S. FDA, 2015, 2016, 2020g). Based on analytical data presented demonstrating that LNnT produced by Inbiose is of equal or greater purity to LNnT preparations that have previously been determined to be GRAS, studies characterizing the safety of LNnT in humans are considered relevant to the safety assessment of Inbiose's ingredient.

The clinical studies in GRN 547, 659, and 919 are briefly summarized below in Table 6.4-1. The results confirm that LNnT is well-tolerated in infants when provided at concentrations within the normal range measured in human milk. LNnT also is well tolerated in health adult subjects. Adverse effects of high intakes of LNnT are similar to those observed with other sources of dietary fiber (*e.g.*, gastrointestinal discomfort) and is self-limiting.

Table 6.4-1 Summary of Human Studies to Support the Safety of Inbiose's LNnT

Type of Study	Population	Length of Study	Dose	Result	Reference
DB, R, PC	228 healthy infants and toddlers (male and female; 6 to 24 months of age)	112 days	An infant formula supplemented with LNnT at a use level of 220 mg/L *ad libitum* Control: Same formula without LNnT	LNnT (produced *via* a yeast fermentation process) was well-tolerated in infants and was without adverse effects on growth and ear health The dose provided is consistent with the lower range of LNnT levels present in mature human breast milk.	Prieto (2005)

Table 6.4-1 Summary of Human Studies to Support the Safety of Inbiose's LNnT

Type of Study	Population	Length of Study	Dose	Result	Reference
DB, R, PC, PD	100 healthy adult volunteers	2 weeks	Single daily doses of LNnT, 2'-FL, or a combination of LNnT and 2'-FL at a ratio of 2:1 at 5, 10, or 20 g/day Single daily dose of 2'-FL or LNnT alone (at 5, 10, or 20 g/day), or a combination of 2'-FL and LNnT (5, 10, or 20 g/day at a ratio of 2:1) Placebo: Glucose	No clinically relevant changes in hematological or biochemical parameters were observed. LNnT was well-tolerated, and no changes in bowel habits *versus* control were noted. The results support that LNnT is safe and well-tolerated in healthy adults.	Elison *et al.* (2016)
Blinded, controlled, R, MC, PD	175 healthy, full-term infants (0 to 6 months of age)	Time of enrollment until 12 months of age	Standard infant formula supplemented with LNnT (at a target concentration of 0.5 g LNnT/L reconstituted formula) in combination with 2'-FL (at a target concentration of 1.0 g/L reconstituted formula)	The study concluded that formula containing LNnT and 2'-FL was safe, well-tolerated, and supported age-appropriate growth	Puccio *et al.* (2017)
Cross-over DBPCFC	67 infants (2 months to 4 years of age) with documented cow milk protein allergy	1 week (7 to 9 days)	A formula supplemented with 0.5 g/L LNnT and 1.0 g/L 2'-FL; minimum of 240 mL daily Control: A formula (hypoallergenic, whey-based, extensively hydrolyzed formula without HMOs)	The results indicated that LNnT does not provoke allergic responses in cow milk protein allergy infants.	Nowak-Wegrzyn *et al.* (2019)

2'-FL = 2'-fucosyllactose; DB = double-blind; DBPCFC = double-blind, placebo-controlled, food challenges; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose; MC = multi-center; PC = placebo-controlled; PD = parallel design; R = randomized.

In addition, 3 new clinical studies of LNnT (provided as a mixture with 2'-FL) were identified in the updated search of the scientific literature for studies published after the submission of GRN 919. One study was conducted in infants (Román Riechmann *et al.*, 2020) and 2 studies were conducted in adult patients with irritable bowel syndrome (IBS) (Iribarren *et al.*, 2020; Palsson *et al.*, 2020). None of the identified studies affect the overall conclusion of safety established in previous GRAS Notices.

In an open label prospective study, healthy term infants (7 days to 2 months of age) were separated into three groups and were exclusively breast fed (n=45), fed commercial 100% whey infant formula containing both 0.5 g/L LNnT and 1.0 g/L 2'-FL (n=63), or fed a mixture of trial formula and breast milk (n=48) for 8 weeks (Román Riechmann *et al.*, 2020). Primarily, measures of growth (*i.e.*, weight, length, and head circumference) and gastrointestinal tolerance (assessed *via* an Infant Gastrointestinal Symptom Questionnaire) were monitored. Any adverse events that occurred throughout the study were also

recorded and assessed for duration, intensity, and frequency. No significant differences were observed in any anthropometric growth measures, gastrointestinal tolerance, or adverse events at the end of the 8-week test period between the 3 infant groups. Infant formula containing 0.5 g LNnT/L (and 1.0 g 2'-FL/L) was reported to be well tolerated by the study authors.

In a study of treatment options for IBS, 245 adults were provided with 5 g of Glycom's 2'-FL/LNnT mixture (97% purity; 4:1 mass ratio) daily over a 12-week intervention period (Palsson *et al.*, 2020). Abnormal bowel movements (as measured by the Bristol Stool Form Scale) were measured as the primary outcome of the study. Severity and/or frequency of abdominal pain, bloating, gastrointestinal symptoms, or IBS-related quality of life was compared to baseline levels for each participant. Any adverse events were also noted throughout the course of the study. Frequency of abnormal stool consistency was significantly decreased from baseline following the 12-week intervention. The severity of IBS symptoms experienced by participants who received the HiMO mixture was also significantly reduced relative to baseline levels. Common side effects of mild gastrointestinal symptoms such as flatulence, abdominal pain and discomfort, and distension were reported throughout the study; however, no serious adverse events were considered to be related to the intervention. The study participants reported that the intervention was well-tolerated, and no safety concerns were identified with its use.

In the subsequent study, the effect of human milk oligosaccharide (Glycom's 2'-FL/LNnT blend) supplementation on adults with IBS was investigated in a parallel, randomized, double-blind, and placebo-controlled study (Iribarren *et al.*, 2020). Sixty patients (male and female) were provided placebo (powdered glucose), 5 g 2'-FL/LNnT, or 10 g 2'-FL/LNnT for daily consumption over a 4-week treatment period. The HiMO mixture contained 2'-FL and LNnT in a 4:1 ratio. Measurements were taken of body weight and height at baseline and upon test completion, and any adverse events or changes in medication and diet were recorded. Patients were also required to complete a questionnaire to assess severity of gastrointestinal and psychological symptoms. No significant differences were observed between treatment groups related to the severity gastrointestinal symptoms or their occurrence. No significant adverse events were reported in the study. Thus, daily intake of 5 and 10 g 2'-FL/LNnT was concluded by the study authors to be well-tolerated by adults with IBS.

6.5 Allergenicity

The potential allergenic activity of the recombinant proteins expressed in *E. coli* K-12 MG1655 INB-LNnT_01 was assessed by using the Allergen Online Tool (V21, released on 14 February 2021) of the University of Nebraska – Lincoln (FARRP, 2021). The database contained 2233 putative allergen sequences. Potential allergenicity was evaluated by scanning each possible 80-amino acid segment of the recombinant protein (sliding window) to the database, and therefore looking for matches of at least 35% identity. No sequence alerts from potential allergens were identified for the recombinant proteins in INB-LNnT_01.

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

6.6 General Recognition

As discussed, the use of LNnT as an ingredient in non-exempt term infant formula at levels up to 0.6 g/L and in various conventional food products has been evaluated by multiple experts, qualified through scientific training and experience, in the safety evaluation of food and infant formula ingredients (GRN 547, 659, 919). The use of LNnT in infant formula at concentrations up to 0.6 g/L and various food products also has been the subject of comprehensive evaluations by multiple authoritative bodies, including EFSA and Food Standards Australia New Zealand (EFSA, 2015, 2020; FSAI, 2016; FSANZ, 2021). As Inbiose has demonstrated that LNnT manufactured by the company is qualitatively and quantitatively highly similar to LNnT ingredients that have been the subject of previous GRAS evaluations and global novel food approvals and is intended for use in the same foods and at the same use levels as those concluded to be GRAS, conclusions on the safety of LNnT for these uses issued by various experts and scientific bodies forms a basis for general recognition of Inbiose's GRAS conclusion. Convening of a GRAS Panel was therefore not considered necessary to support a GRAS conclusion on the basis that this HMO ingredient has been evaluated by multiple GRAS Panels and authoritative bodies, including the U.S. FDA and EFSA.

6.7 Conclusion

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

LNnT therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the *Code of Federal Regulations*.

Part 7. § 170.255 List of Supporting Data and Information

Asakuma S, Hatakeyama E, Urashima T, Yoshida E, Katayama T, Yamamoto K, et al. (2011). Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. J Biol Chem 286(40):34583-34592 [plus supplementary data]. DOI:10.1074/jbc.M111.248138.

Asakuma S, Urashima T, Akahori M, Obayashi H, Nakamura T, Kimura K, et al. (2008). Variation of major neutral oligosaccharides levels in human colostrum. Eur J Clin Nutr 62(4):488-494. DOI:10.1038/sj.ejcn.1602738.

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

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

Bao Y, Chen C, Newburg DS (2013). Quantification of neutral human milk oligosaccharides by graphitic carbon high-performance liquid chromatography with tandem mass spectrometry. Anal Biochem 433(1):28-35. DOI:10.1016/j.ab.2012.10.003.

Bentz S (2021) [unpublished]. *Lacto-N-neoTetraose (LNnT) - Preliminary Toxicity Study by Oral Route (Gavage) in Juvenile Rats: Final Report*. (Study No. 48336 RSR). Charles River Laboratories Evreux.

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

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

CDC (2011). N*ational Health and Nutrition Examination Survey (NHANES): 2009-2010*. Hyattsville (MD): Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://www.cdc.gov/nchs/nhanes/nhanes20092010/nhanes09_10.htm [Page last updated: November 7, 2011]. Cited In: Glycom A/S, 2014 [GRN 547].

CDC (2015). National Health and Nutrition Examination Survey (NHANES): 2011-2012. Hyattsville (MD), Centers for Disease Control and Prevention (CDC), National Center for Health Statistics (NCHS). Available at: http://wwwn.cdc.gov/nchs/nhanes/search/nhanes11_12.aspx [Page last reviewed: November 6, 2015]. Cited In: Glycom A/S, 2016 [GRN 659].

Chaturvedi P, Warren CD, Altaye M, Morrow AL, Ruiz-Palacios G, Pickering LK, et al. (2001). Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology 11(5):365-372. DOI:10.1093/glycob/11.5.365.

Chaturvedi P, Warren CD, Ruiz-Palacios GM, Pickering LK, Newburg DS (1997). Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives. Anal Biochem 251(1):89-97. DOI:10.1006/abio.1997.2250.

Cheng L, Akkerman R, Kong C, Walvoort MTC, de Vos P (2021). More than sugar in the milk: human milk oligosaccharides as essential bioactive molecules in breast milk and current insight in beneficial effects. Crit Rev Food Sci Nutr 61(7):1184-120. DOI:10.1080/10408398.2020.1754756.

Coppa GV, Pierani P, Zampini L, Carloni I, Carlucci A, Gabrielli O (1999). Oligosaccharides in human milk during different phases of lacation. Acta Paediatr 88(Suppl. 430):89-94. DOI:10.1111/j.1651-2227.1999.tb01307.x.

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

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

EFSA (2015). Scientific opinion on the safety of lacto-*N*-neotetraose as a novel food ingredient pursuant to Regulation (EC) No 258/97. (EFSA Panel on Dietetic Products, Nutrition and Allergies/NDA) (Question no EFSA-Q-2014-00862, adopted: 29 June 2015 by European Food Safety Authority). EFSA J 13(7):4183 [32pp]. DOI:10.2903/j.efsa.2015.4183. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4183.

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

EFSA (2020). Safety of lacto-*N*-neotetraose (LNnT) produced by derivative strains of *E. coli* BL21 as a novel food pursuant to Regulation (EU) 2015/2283 (EFSA Panel on Nutrition, Novel Foods and Food Allergens/NDA) (Question number: EFSA-Q-2019-00448, adopted: 22 October 2020 by European Food Safety Authority). EFSA J 18(11):6305 [11pp]. DOI:10.2903/j.efsa.2020.6305. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/6305.

Elison E, Vigsnaes LK, Rindom Krogsgaard L, Rasmussen J, Sørensen N, McConnell B, et al. (2016). Oral supplementation of healthy adults with 2'-*O*-fucosyllactose and lacto-*N*-neotetraose is well tolerated and shifts the intestinal microbiota. Br J Nutr 116(8):1356-1368 [plus supplementary table]. DOI:10.1017/S0007114516003354.

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

Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O'Ryan ML, et al. (2000). Variability of human milk neutral oligosaccharides in a diverse population. J Pediatr Gastroenterol Nutr 30(2):181-192. DOI:10.1097/00005176-200002000-00016.

FARRP (2021). *AllergenOnline Version 21: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org [Released: February 14, 2021].

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

FSAI (2016). *Substantial Equivalence Opinion: Lacto-N-neotetraose Produced by Microbial Fermentation (LNnT).* [Applicant Glycom A/S]. (EC No. 359). Dublin, Ireland: Food Safety Authority of Ireland (FSAI). Available at: https://www.fsai.ie/uploadedFiles/Science_and_Health/Novel_Foods/Notifications/SE%20opinion%20LNnt.pdf.

FSANZ (2021). *A1155 – 2'-FL and LNnT in Infant Formula and Other Products*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/code/applications/Pages/A1155.aspx [Page last updated: 8 April 2021].

Gabrielli O, Zampini L, Galeazzi T, Padella L, Santoro L, Peila C, et al. (2011). Preterm milk oligosaccharides during the first month of lactation. Pediatrics 128(6):e1520-e1531. DOI:10.1542/peds.2011-1206.

Galeotti F, Coppa GV, Zampini L, Maccari F, Galeazzi T, Padella L, et al. (2012). On-line high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry profiling of human milk oligosaccharides derivatized with 2-aminoacridone. Anal Biochem 430(1):97-104 [plus supplementary Appendix A]. DOI:10.1016/j.ab.2012.07.027.

Glycom A/S (2014). *GRAS Exemption Claim for Lacto-N-neotetraose*. (GRN No. 547). Submitted by Kgs. Lyngby, Denmark, Glycom A/S to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=547 [Oct. 2, 2015 - FDA response - no questions].

Glycom A/S (2016). *GRAS Exemption Claim for Lacto-N-neotetraose*. (GRN No. 659). Submitted by Lyngby, Denmark: Glycom A/S to College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=659 [Nov. 23, 2016 - FDA response - no questions].

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

Iribarren C, Törnblom H, Aziz I, Magnusson MK, Sundin J, Vigsnaes LK, et al. (2020). Human milk oligosaccharide supplementation in irritable bowel syndrome patients: a parallel, randomized, double-blind, placebo-controlled study. Neurogastroenterol Motil 32(10):e13920 [12pp, plus supplementary data]. DOI:10.1111/nmo.13920.

Leo F, Asakuma S, Fukuda K, Senda A, Urashima T (2010). Determination of sialyl and neutral oligosaccharide levels in transition and mature milks of Samoan women, using anthranilic derivatization followed by reverse phase high performance liquid chromatography. Biosci Biotechnol Biochem 74(2):298-303. DOI:10.1271/bbb.90614.

Levin S, Semler D, Ruben Z (1993). Effects of two weeks of feed restriction on some common toxicologic parameters in Sprague-Dawley rats. Toxicol Pathol 21(1):1-14. DOI:10.1177/019262339302100101.

Nakhla T, Fu D, Zopf D, Brodsky NL, Hurt H (1999). Neutral oligosaccharide content of preterm human milk. Br J Nutr 82(5):361-367. DOI:10.1017/S0007114599001609.

Nowak-Wegrzyn A, Czerkies L, Reyes K, Collins B, Heine RG (2019). Confirmed hypoallergenicity of a novel whey-based extensively hydrolyzed infant formula containing two human milk oligosaccharides. Nutrients 11(7):1447 [10pp]. DOI:10.3390/nu11071447.

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

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

OECD (1997b). *In vitro* mammalian cell gene mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 476) [updated & adopted: 21st July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-476-in-vitro-mammalian-cell-gene-mutation-tests-using-the-hprt-and-xprt-genes_9789264264809-en;jsessionid=58ip5ibjcf4kc.x-oecd-live-02 [Latest version updated & adopted: 29 July 2016].

OECD (1998). Repeated dose 90-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline No. 408) [Updated & Adopted: 21 September 1998]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-408-repeated-dose-90-day-oral-toxicity-study-in-rodents_9789264070707-en;jsessionid=1m6bvwspup322.delta [New Version Updated & Adopted: 27 June 2018].

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

OECD (2008b). Repeated dose 28-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 407) [Adopted: 3 Oct 2008]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: http://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en.

Orosz I (2021) [unpublished]. *A GLP Acute Oral Toxicity Study of Lacto-N-neoTetraose in Rats (Up and Down Procedure): Final Report*. (Study No. 20/068-001P). Charles River Laboratories Hungary Kft.

Palsson OS, Peery A, Seitzberg D, Amundsen ID, McConnell B, Simrén M (2020). Human milk oligosaccharides support normal bowel function and improve symptoms of irritable bowel syndrome: a multicenter, open-label trial. Clin Transl Gastroenterol 11(12):e00276 [7pp, plus supplementary figures]. DOI:10.14309/ctg.0000000000000276.

Penard L (2016). *LNnT – 13-Week Oral (Gavage) Juvenile Toxicity Study in the Rat Followed by a 4-Week Treatment-free Period*. (Study No.: AB20758, Sponsor Ref. No.: GSN041). Prepared by Saint-Germain-Nuelles, France: WIL Research Europe-Lyon for Lyngby, Denmark: Glycom. Cited In: Glycom A/S, 2016 [GRN 659].

Prieto PA (2005). *In vitro* and clinical experiences with a human milk oligosaccharide, lacto-N-*neo*Tetrose, and fructooligosaccharides. FFI J 210(11):1018-1030.

Puccio G, Alliet P, Cajozzo C, Janssens E, Corsello G, Sprenger N, et al. (2017). Effects of infant formula with human milk oligosaccharides on growth and morbidity: a randomized multicenter trial. J Pediatr Gastroenterol Nutr 64(4):624-631. DOI:10.1097/MPG.0000000000001520.

Román Riechmann E, Moreno Villares JM, Domínguez Ortega F, Carmona Martínez A, Picó Sirvent L, Santana Sandoval L, et al. (2020). Real-world study in infants fed an infant formula with two human milk oligosaccharides. Nutr Hosp 37(4):698-706. DOI:10.20960/nh.03084.

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

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

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

Sumiyoshi W, Urashima T, Nakamura T, Arai I, Saito T, Tsumura N, et al. (2003). Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. Br J Nutr 89(1):61-69. DOI:10.1079/BJN2002746.

Thurl S, Henker J, Siegel M, Tovar K, Sawatzki G (1997). Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. Glycoconj J 14(7):795-799. DOI:10.1023/A:1018529703106.

Thurl S, Munzert M, Henker J, Boehm G, Müller-Werner B, Jelinek J, et al. (2010). Variation of human milk oligosaccharides in relation to milk groups and lactational periods. Br J Nutr 104(9):1261-1271. DOI:10.1017/S0007114510002072.

Tóth-Gönczöl K (2021) [unpublished]. *GLP Bacterial Reverse Mutation Assay of Lacto-N-neoTetraose: Final Report*. (Study No. 0/068-007M). Charles River Laboratories Hungary Kft.

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

U.S. FDA (2015). *Agency Response Letter GRAS Notice No. GRN 547 [Lacto-N-neotetraose, Lyngby, Denmark: Glycom A/S*]. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=547 [Oct. 2, 2015 - FDA response - no questions].

U.S. FDA (2016). *Agency Response Letter GRAS Notice No. GRN 659 [Lacto-N-neotetraose, Lyngby, Denmark: Glycom A/S]*. College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=659 [Nov. 23, 2016 - FDA response - no questions].

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

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

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

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

U.S. FDA (2020d). *Agency Response Letter GRAS Notice No. GRN 897 [2'-O-fucosyllactose, Wilmington (DE): DuPont Nutrition and Health]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=897 [Feb. 24, 2020; Correction: Apr. 13, 2020 - FDA response - no questions].

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

U.S. FDA (2020f). *Agency Response Letter GRAS Notice No. GRN 895 [Lacto-N-neotetraose: Hørsholm, Denmark: Glycom A/S]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=895 [Pending as of Feb. 28, 2020].].

U.S. FDA (2020g). *Agency Response Letter GRAS Notice No. GRN 919 [Preparation Lacto-N-neotetraose, Reinbreitbach, Germany: Jennewein Biotechnologie GmgH]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=919 [Oct. 30, 2020 - FDA response - no questions].

USDA (2012). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2009-2010*. Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release [Last Modified: 07/16/2012]. Cited In: Glycom A/S, 2014 [GRN 547].

USDA (2014). *What We Eat in America: National Health and Nutrition Examination Survey (NHANES): 2011-2012*. Riverdale (MD): U.S. Department of Agriculture (USDA). Available at: http://www.ars.usda.gov/Services/docs.htm?docid=13793#release [Last Modified: November 2, 2014]. Cited In: Glycom A/S, 2016 [GRN 659].

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

Verbaan IAJ (2016). *An In Vitro Micronucleus Assay with LNNT in Cultured Peripheral Human Lymphocytes*. (Laboratory Project Identification: Project 507474; Substance 206095/B). Prepared by DD's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark: Glycom. Cited In: Glycom A/S, 2016 [GRN 659].

Verchère-Beau (draft report due September 2021) [unpublished]. *13-week Juvenile Toxicity Study of Lacto-N-neotetraose by the Oral (gavage) Route in the Rat with a 4-week Recovery Period. Draft Report*. (Study No. 20283119). Charles River Laboratories France Safety Assessment SAS, Saint-Germain-Nuelles.

Verspeek-Rip CM (2016). *Evaluation of the Mutagenic Activity of LNNT in the Salmonella Typhimurium Reverse Mutation Assay and the Escherichia Coli Reverse Mutation Assay (Plate Incorporation and Pre-incubation Methods)*. (Laboratory Project Identification: Project 507473; Substance 206095/B). Prepared by DD's-Hertogenbosch The Netherlands: WIL Research Europe B.V. for Lyngby, Denmark: Glycom. Cited In: Glycom A/S, 2016 [GRN 659].

Williams JE, Price WJ, Shafii B, Yahvah KM, Bode L, McGuire MA, McGuire MK (2017). Relationships among microbial communities, maternal cells, oligosaccharides, and macronutrients in human milk. J Hum Lact 33(3):540-551. DOI:10.1177/0890334417709433.

January 04, 2023

Ellen T. Anderson
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Regarding: Response to FDA Questions related to GRAS Notice No. GRN 001067

Dear Dr. Anderson,

In reference to your letter dated December 12, 2022, regarding Inbiose's GRAS notice GRN 001067 for the intended uses of lacto-*N*-neotetraose (LNnT), I am pleased to provide you with our responses to the Agency's questions in the following document.

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

Kind regards,

Joeri Beauprez
Chief Scientific Officer

Question 1. *The intended uses of LNnT described in the notice include use in non-exempt infant formula for term infants. Please clarify the intended source of the infant formula protein base (e.g., milk, soy, whey) into which LNnT would be added.*

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

Question 2. *Please state whether any of the raw materials used in the fermentation are major allergens or derived from major allergens. If any of the raw materials used are major allergens or derived from major allergens, please discuss why these materials do not pose a safety concern.*

Inbiose's LNnT is produced using milk-derived lactose. As such, any products that include this ingredient would be required to include "contains milk" on the label in accordance with the requirements of the Food Allergy, Labelling and Consumer Protection Act (FALCPA) of 2004. None of the other raw materials used in the fermentation are themselves considered, or are derived from, major allergens as defined by FALCPA (*i.e.*, milk, egg, fish, Crustacea shellfish, tree nuts, wheat, peanuts, and soybeans), or sesame.

Question 3. *For the administrative record, please briefly specify how the purity and genetic stability of the host culture is ensured. Please also briefly specify any controls used during fermentation and state whether the fermentation process is conducted in a contained, sterile environment.*

The overall fermentation process used in the production of Inbiose's LNnT is conducted within a contained, sterile, environment, and the use of strict process controls ensures that the purity and genetic makeup of the host culture remains stable throughout fermentation.

Once the identity of the INB-LNnT_01 production strain was originally established, a batch of cryovials was collected from the strain for use for LNnT production. The following quality checks are performed on randomly selected cryovials of the cryovial batch to ensure purity and genetic uniformity of the production strain prior to use in the fermentation process:

- Inoculation and growth on Lysogeny broth (LB) and Minimal medium, followed by the extraction of genomic DNA for Whole Genome Sequencing (WGS) *via* the Illumina platform (*i.e.*, 150 bp paired-end sequencing), to monitor genetic uniformity and purity of the collected cryovials
- Polymerase chain reaction (PCR) checks to confirm the presence of all integrated genes in the collected samples
- Growth in a shake flask with minimal medium, was followed by a gram-staining of the production organism, measurement of sugar production and the optical density at 600 nm.
- Measurement of colony forming unit (CFU) counts and colony morphology checks. Random colonies are selected from all plated samples and integrated genes are checked *via* PCR. A growth experiment is also performed with random selected colonies from all plated samples to evaluate the growth speed (μ_{max}) and LNnT production after 72 hours of growth.

After the cryovial quality is verified, fermentations with INB-LNnT_01 are performed using cryovials from the batch as an inoculum.

As indicated in the GRAS Notice Section 2.2.1.2, the "*production strain INB-LNnT_01 proved to be 100% stable within the production environment after analysis by next generation sequencing of samples at the end of fermentation at pilot scale.*" This purity and genetic stability analysis of the INB-LNnT_01 strain host culture was conducted post-fermentation, following five non-consecutive fed-batch fermentations, using WGS of genomic DNA obtained from the residual biomass at the end of each fermentation. Each of the tested samples of residual biomass correspond to the five batches of LNnT presented in Section 2.3.2 of the GRAS Notice. Results from the WGS of post-production biomass were compared with results from an overnight LB culture of the production strain, INB-LNnT_01. No evidence of significant mutation was observed in any of the five samples collected post-fermentation relative to the production strain INB-LNnT_01. All mutations identified in post-fermentation samples were comparable to those of the INB-LNnT_01 strain reference from the initial cryovials. Overall, these analyses support that the genetic stability and purity of the production strain is maintained throughout fermentation.

Question 4. *For the administrative record, please specify that the production organism is non-pathogenic and non-toxigenic.*

As indicated in Subsection 2.2.1.1 of the GRAS Notice, the host organism, *E. coli* K-12 MG1655, is not considered a human or animal pathogen and is non-toxigenic. As this organism is classified as Biosafety Level 1 classification by the American Type Culture Collection (ATCC), and meets the Organisation for Economic Co-operation and Development (OECD)'s Good Industrial Large-Scale Practice (GILSP) criteria for working with genetically modified microorganisms (OECD, 1992).

Inbiose hereby confirms that the LNnT ingredient subject to this GRAS notification is produced using a non-pathogenic and non-toxigenic production organism.

Question 5. *On page 36 of the notice, Inbiose states, "Adverse effects of high intakes of LNnT are similar to those observed with other sources of dietary fiber (e.g. gastrointestinal discomfort) and is self-limiting." Please explain this statement. We note that FDA has not determined whether LNnT meets the statutory definition of "dietary fiber", and thus LNnT or any human milk oligosaccharide (HMO) would not be labeled as such at this time. Therefore, it is not clear that consumers would be aware that high intake of HMOs or LNnT in particular would be expected to lead to potential gastrointestinal discomfort and that the intake will necessarily be self-limiting.*

Thank you for providing us with the opportunity to bring clarity regarding this sentence—the way it is presented is indeed incorrect and was not intended to imply that the HMO, LNnT, is a dietary fiber ingredient. Instead, the purpose of this statement was to highlight the parallel between the self-limiting nature of LNnT and other ingredients, such as dietary fibers, which are considered self-limiting due to the potential for gastrointestinal discomfort to occur at the highest levels of intake. While supplementation of LNnT has been exhibited as safe and well tolerated, and no LNnT-related adverse effects were noted up to the highest tested doses in a wide range of animal studies and clinical trials (Pages 24-38 of the GRAS Notification), sporadic individual gastrointestinal symptoms, such as an increase in passing gas and bloating, have been observed at some of the

highest dose levels tested in humans (Elison *et al.*, 2016). These effects, while not considered to be adverse, were highlighted as symptoms that might cause gastrointestinal discomfort. Even so, similar rates of adverse effect occurrence have been identified across HMO-test formula, breast fed, and controlled reference groups in clinical trials.

> Rewritten, the identified sentence from page 36 of the Notice could be rewritten, as follows: "*Gastrointestinal effects, such as an increase in bloating and passing gas, may occur at high intake levels of LNnT, which could be perceived as gastrointestinal discomfort in some individuals and therefore be viewed as self-limiting.*"

Question 6. *On page 23 of the notice, Inbiose states that a literature search was conducted through April 2021. Please confirm that no new publicly available data have been found since that date that could be perceived as counter to your GRAS conclusion. If such data is found, please discuss their implications on your GRAS conclusion.*

To address this question, Inbiose has conducted an updated literature search through December 2022 to identify any new publicly available data pertaining to the safety of LNnT that have been published since the original literature search was conducted in April 2021. No new data were identified in the updated search of the published literature that could be perceived as counter to Inbiose's LNnT GRAS conclusion; however, several new clinical studies were identified in support of the GRAS conclusion. While the results from these studies are not counter to the GRAS conclusion, these studies are summarized in Table 1, below, for completeness. Briefly, LNnT (in combination with 2'-fucosyllactose, another HMO ingredient) was not observed to elicit adverse effects in humans. Results from these recently published studies support that LNnT is safe and well-tolerated in infants when provided at levels consistent with the proposed uses of Inbiose's LNnT described in the GRAS Notification. Inbiose therefore maintains that this LNnT ingredient is GRAS, on the basis of scientific procedures, for use in non-exempt term infant formula and specified conventional food and beverage products, as described in the GRAS Notification.

Table 1 -- Summaries of Newly Identified Clinical Trials Conducted with LNnT in Combination with 2'-FL

Type of Study	Population	Length of Study	Dose	Result	Reference
Parallel, double-blind, randomized, placebo-controlled study. Exploratory study, please refer to Iribarren *et al.* (2020)	Adult IBS patients	4 weeks	5 g of 2'-FL (N=20) or 10 g of 2'-FL and LNnT mix at ratio of 4:1 (N = 20) Placebo: glucose	Supplementation with 2'-FL/LNnT modulated the gut microbiota, fecal and plasma metabolite profiles, but not the host mucosal response in IBS. Furthermore, the bifidogenic effect was associated with metabolite modulation.	Iribarren *et al.* (2021)
Randomized, double-blinded, placebo-controlled trial	Children with overweight (including obesity) ages 6 to 12 years	8 weeks	4.5 g of 2'-FL (N = 21) 4.5 g of 2'-FL and LNnT in a 4:1 mass ratio (N = 24) Placebo: powdered glucose (N = 18)	The relative abundance of bifidobacteria increased after 4 weeks (p = 0.033) in the Mix-group, no change was observed in the placebo group. No serious AEs were reported.	Fonvig *et al.* (2021)
Controlled, double-blind, randomized clinical trial	Full-term infants aged 0–6 months with physician-diagnosed CMPA were enrolled	Time of enrolment until 12 months of age	100% whey-based EHF supplemented with 2'-FL and LNnT (1.0 g/L and 0.5 g/L, respectively, N = 71). Placebo: EHF without HMO (N = 71).	The test formula with HMOs was well tolerated with a safety profile comparable EF formula without HMO. There were no significant differences in anthropometric parameters between the groups. There was a statistically significant reduction in the frequency of upper respiratory tract infections and a lower incidence of ear infections at 12 months in test formula with HMOs. HMO-supplemented formula supports normal growth in infants with CMPA	Vandenplas *et al.* (2022)

Table 1 -- Summaries of Newly Identified Clinical Trials Conducted with LNnT in Combination with 2'-FL

Type of Study	Population	Length of Study	Dose	Result	Reference
Prospective, randomized, double-blind, controlled trial	Pre-term infants (27–33 weeks' gestation, birth weight <1,700 g) and younger than 7 days of age were enrolled into the study	Time of enrollment within 7 days of birth until discharge from the neonatal unit	2'-FL and LNnT in a 10:1 ratio (0.374 and 0.034 g/kg bw/day, respectively) (N=43) Placebo: consisting of only glucose (0.140 g/kg bw/day) (N = 43)	No significant difference between the adjusted mean time to reach FEF from birth; Head circumference-for-age z-score was higher in HMO *vs*. placebo at discharge (p = 0.007). Gastric residual volume was low in both groups. There was comparable average stool consistency, number of stools per day. Occurrence of AEs was similar in both groups.	Hascoët *et al.* (2022)
Open-label, non-randomized, multicenter trial	Term infants aged 1–8 months with moderate-to-severe CMPA	Time of enrolment until 12 months of age	Amino acid-based formula supplemented with 2'-FL and LNnT (1.0 g/L and 0.5 g/L, respectively) (N = 29)	Fecal microbiome changes (significant on-treatment enrichment in HMO-utilizing bifidobacteria and significant reduction in the abundance of faecal Proteobacteria when compared to baseline). HMO-supplemented study formula was safe and well tolerated.	Gold *et al.* (2022)

2'-FL = 2'-fucosyllactose; AE = adverse event; bw = body weight; CMPA = cow's milk protein allergy; EHF = extensively hydrolysed formula; FEF = full enteral feeding; HMO = human milk oligosaccharide; IBS = irritable bowel syndrome; LNnT = lacto-*N*-neotetraose; N = number of participants.

Question 7. *On page 31 of the notice, Inbiose states, "Currently, a 90-day study is being conducted to evaluate the potential toxic effects of Inbiose's LNnT on the development of juvenile rats, following daily oral administration, from PND 7 to at least PND 97 (Verchère-Beau, 2021 [unpublished draft report]). This study is ongoing, and a draft report is not available at the time of submitting this notice. The results of this study will be provided as supplemental information to the notice once available." Furthermore, in the notice's Part 7, List of Supporting Data and Information, on page 46, it states that the unpublished draft report was due September 2021.*

It appears Inbiose's GRAS conclusion was made without knowing the results of this 90-day study. Please provide the summary of the results and discuss whether the conclusion from this study support your GRAS conclusion. We note that since you have acknowledged to us the existence of this study, we will not be able to complete our evaluation until we know its outcome and conclusion.

Following the filing of this GRAS Notice, the 90-day study was completed. Inbiose conducted this 90-day subchronic study in juvenile rats to corroborate the safety of LNnT and to support premarket approvals in global jurisdictions where such studies may be necessary (Verchere Beau, 2022 [final report, unpublished]). This study was conducted in accordance with the 2018 OECD Test Guideline 408 and not the 1998 OECD Test Guideline, as was erroneously reported in the heading of Section 6.3.1.4. In the main study, groups of 20 (10/sex/group) juvenile Sprague-Dawley rats were administered Inbiose´s LNnT at dose levels of 0, 1,500, 3,000, or 5,000 mg/kg body weight/day *via* oral gavage, from post-natal day (PND) 7 to at least PND 97. An additional group was included for reference, in which a group of 20 (10/sex) juvenile Sprague-Dawley rats were administered 5,000 mg fructooligosaccharides (FOS)/kg body weight/day. The control and high-dose groups also included 10 additional rats (5/sex/group) as recovery animals, and another 6 animals (3/sex/group) were included for toxicokinetic evaluation in each of the 0, 1,500, 3,000, or 5,000 mg LNnT/kg body weight/day treatments.

The following parameters and end points were evaluated in this study during the dosing period: clinical observations, body weights, food consumption, growth (tibia length), ophthalmology, developmental pre-weaning end points, sexual maturation, estrous cycles, and neuro-behavioral development (behavioral functional observational battery, learning and memory retention, and locomotor activity), clinical pathology (hematology, coagulation, clinical chemistry, and urinalysis), thyroid hormone levels, gross necropsy findings, organ weights, sperm analysis data and histopathologic findings. At the end of the 13-week treatment period, urine and plasma samples were collected for toxicokinetic evaluation.

No LNnT or FOS-related clinical signs, adverse effects, or macroscopic and microscopic changes were observed following oral administration. The study researchers reported that Inbiose's LNnT was "*...well tolerated, with no remarkable effect on development and reproductive function and no neurotoxicity*" and a "*Slight non adverse reduction in triglyceride was noted for both sexes.*" The no-observed-adverse-effect level (NOAEL) in this study was therefore established by the study director as 5,000 mg/kg body weight/day for both males and females, which supports Inbiose's GRAS conclusion that LNnT is safe for use in non-exempt term infant formula and specified conventional food and beverage products as described in Section 1.3 of the GRAS Notice.

For the sake of completeness, Inbiose is also providing the U.S. FDA with the summary of this study, which was produced by the contract research organization, and is included in the attached pdf document entitled: **"Supplementary 90-day Subchronic Study Summary".**

2. SUMMARY

The objectives of this study were to determine the potential toxicity of Lacto-N-neoTetraose (LNnT) in the juvenile rat following daily oral administration from Post Natal Day 7 (PND7) to at least PND97 (13-week treatment period), with monitoring of subsequent development and reproductive function, an evaluation of potential LNnT-related neurotoxicity and an assessment of systemic exposure under the defined experimental conditions. On completion of the dosing period, the regression/reversibility of any LNnT-related effects after at least 4 weeks of a treatment-free period was evaluated on designated animals (control and high dose level groups).

The study design was as follows:

Text Table 1
Experimental Design

Group No.	Test Material	Dose Level (mg/kg/day)	Dose Volume[a] (mL/kg)	Dose Concentration (mg/mL)	Target Number of Animals					
					Main Study		Recovery Study		Toxicokinetic Study	
					M	F	M	F	M	F
1	Control item[b]	0	10	0	10	10	5	5	3	3
2	LNnT	1500	10	150	10	10	-	-	3	3
3	LNnT	3000	10	300	10	10	-	-	3	3
4	LNnT	5000	10	500	10	10	5	5	3	3
5	FOS[c]	5000	10	500	10	10	-	-	-	-

-: Not applicable; M: Males; F: Females.
[a]: Based on the most recent body weight measurement.
[b]: Control item: Purified water.
[c]: FOS: Reference item (i.e., Actilight 950P).

The following parameters and end points were evaluated in this study during the dosing period: Dose analysis, clinical observations, body weights, food consumption, growth (tibia length), ophthalmology, developmental pre-weaning end points, sexual maturation, estrous cycles, and neuro-behavioral development (behavioral functional observational battery, learning and memory retention and locomotor activity), clinical pathology (hematology, coagulation, clinical chemistry and urinalysis), thyroid hormone levels, gross necropsy findings, organ weights, sperm analysis data and histopathologic findings. At the end of the 13-week treatment period, urine and plasma samples were collected for toxicokinetic evaluation.

The following parameters and end points were evaluated in this study during the 4-week treatment-free period: Clinical observations, body weights, food consumption, clinical pathology (hematology, coagulation and clinical chemistry), thyroid hormone levels, gross necropsy findings, organ weights and histopathologic findings.

Actual concentrations of LNnT in the dose formulations analyzed during the treatment period remained within the defined acceptable range when compared to the nominal concentrations. No LNnT was detected in the control item samples.

No sample contamination with LNnT was noted in control samples (Group 1) as plasma and urine concentrations were below the Lower Limit Of Quantification (LLOQ of 40 ng/mL for plasma and 500 ng/mL for urine) or within the baseline concentration observed in untreated rats (Vazquez et al. 2017) (9). The measurable LNnT concentration in plasma or in urine observed in control samples was considered as an ex-vivo bioanalytical artefact, endogenous level or due to the overestimation following the storage period and/or QC accuracy issues.

LNnT concentrations in plasma were quantifiable in general up until 6 hours postdose of LNnT. The maximum plasma concentration of LNnT was observed between 1 and 2 hours postdose in both genders. The female-to-male ratios ranged from 0.866 to 1.32 for C_{max} and from 0.630 to 0.961 for AUC_{tlast}. The exposure (C_{max} and AUC_{tlast}) increased proportionally with the dose levels in the range of administered doses.

In addition, LNnT concentrations were observed in all urine samples in the interval of 0-24 hours on Study Day 90. The amount of LNnT recovered in urine (as the percentage of the doses) during the 24-hour exposure was low, i.e., < 0.5 % for both males and females.

No LNnT or FOS-related death occurred during the study.

There were no adverse LNnT-related clinical signs at any dose. Hypersalivation was occasionally noted for males at 5000 mg/kg/day.

In all FOS-treated animals, soft to liquid feces associated with dryness, reddish and/or swollen anus together with traces of blood (red discharge) and ungroomed fur were observed for both sexes from Study Day 2 (PND8) up to weaning. In view of the severity of the signs, requiring veterinarian care, these findings were considered as adverse.

There were no relevant LNnT-related effects on mean body weight, mean body weight gain and mean food consumption in any group for either sex during the dosing and 4-week treatment-free periods.

Dosing with FOS was associated with a statistically significant lower mean body weight gain during the first few days of dosing (Study Days 1 to 5) for both sexes and with a slightly lower mean body weight gain for males during the overall dosing period but with no impact on terminal mean body weight; the effect was therefore considered as non adverse. There was no relevant FOS-related effect on mean food consumption for either sex.

There was no relevant LNnT or FOS-related effect on long bone (tibia) growth, developmental landmarks (assessed by incisor eruption, eye opening, righting and gripping reflexes and pupillary and auditory responses), neurologic development (assessed by functional observation battery, motor activity, learning and memory), ophthalmology, estrous cycle or seminology parameters.

There was no relevant LNnT or FOS-related effects on the hematology, coagulation factors and urinalysis parameters or on thyroid hormone levels.

Dosing with LNnT or FOS was associated with non adverse reductions in triglyceride concentration at all doses for both sexes. Non adverse reduction in albumin concentration of males were noted at 5000 mg/kg/day and non adverse increase in glucose concentration for females dosed with 3000 and 5000 mg/kg/day, at the end of the dosing period and/or at the end of the 4-week treatment-free period for which a test item related effect could not be excluded. Yet, these effects were limited to only 1 sex.

At the end of the dosing period, there was no organ weight, macroscopic or microscopic changes related to LNnT or FOS.

In conclusion, the test item, Lacto-N-neoTetraose (LNnT) administered once daily by oral gavage for 13 weeks to juvenile Sprague Dawley rats from PND7, at dose levels of 1500, 3000 or 5000 mg/kg/day was well tolerated, with no remarkable effect on development and reproductive function and no neurotoxicity. Slight non adverse reduction in triglyceride was noted for both sexes.

The reference item, Actilight 950P (FOS) administered following the same dosing regimen at a dose of 5000 mg/kg/day was associated with a transient reduction in body weight gain and changes in fecal appearance during the pre-weaning period together with marked local reactions requiring veterinarian care. After weaning, FOS was well tolerated, with no remarkable effect on development and reproductive function and no neurotoxicity. Slight changes amongst the clinical chemistry parameters were observed, similar to those observed for LNnT, but with no histological correlate.

Based on these results, the No Observed Adverse Effect Level (NOAEL) was established at 5000 mg/kg/day for juvenile males (mean plasma $AUC_{0\text{-tlast}}$ = 19200 ng.h/mL) and females (mean plasma $AUC_{0\text{-tlast}}$ = 12100 ng.h/mL), based on the absence of adverse effects following dosing with LNnT at this dose level.