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

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

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

DATE:
30 June 2021

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

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Part 1. § 170.225 Signed Statements and Certification

In accordance with 21 CFR §170 Subpart E consisting of §170.203 through §170.285, Inbiose N.V. (Inbiose) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of lacto-*N*-tetraose (LNT), 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 LNT 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 LNT 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*-tetraose; LNT

1.3 Conditions of Use

Inbiose's LNT is intended for use as an ingredient in non-exempt infant formula for term infants at a maximum level of 0.8 g/L, and in toddler beverages and other drinks for young children at a maximum level of 0.6 g/L. LNT 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 2.0 g/L, in grain products, pastas, and infant and toddler foods at levels up to 20 g/kg. As this ingredient would serve as an alternative source of LNT, additive increases in LNT consumption are not expected to occur. Use levels and food categories are incorporated by reference to Table 1.3-1 of GRN 833 (replicated in Table 1.3-1 below) and are fully substitutional to those described in the Notice (GRN 833; U.S. FDA, 2019).

Table 1.3-1 Intended Food Uses and Use Levels for LNT in the U.S. (Adapted from GRN 833)

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Proposed Food Use	RACC[a] (g or mL)	Proposed Maximum Use Level (g/RACC)	Proposed Maximum Use Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction[b]	240 mL	0.48	2.0
	Sports and Isotonic Drinks, Energy Drinks, Soft Drinks, Enhanced or Fortified Waters, Fruit-based Ades	360 mL	0.36	1.0
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.08	0.8
	Toddler Formulas[d]	100 mL[c]	0.06	0.6
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.04 to 0.85	5.0
	Other Drinks for Young Children	120 mL	0.07	0.6
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	40 g	0.8	20
	Cereal and Granola Bars	40 g	0.4	10
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk*	240 mL	0.24	1.0
Milk Products	Buttermilk*	240 mL	0.24	1.0
	Flavored Milk	240 mL	0.24	1.0
	Milk-Based Meal Replacement Beverages, for Weight Reduction[b]	240 mL	0.48	2.0
	Yogurt*	170 g	1.7	10

CFR = *Code of Federal Regulations*; GRN = GRAS Notice; LNT = lacto-*N*-tetraose; RACC = Reference Amounts Customarily Consumed per Eating Occasion; U.S. = United States.

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

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

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

[d] Formula/beverage products intended for toddlers >12 months of age.

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

1.4 Basis for GRAS

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

1.5 Availability of Information

The data and information that serve as the basis for this GRAS 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

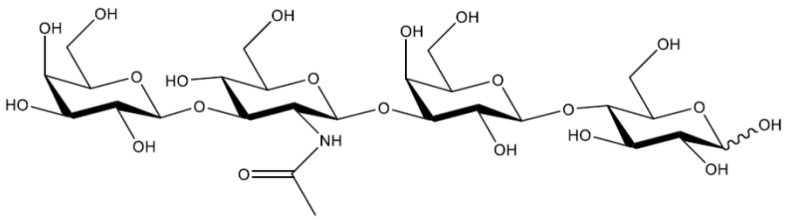
It is Inbiose's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the *Freedom of Information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

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

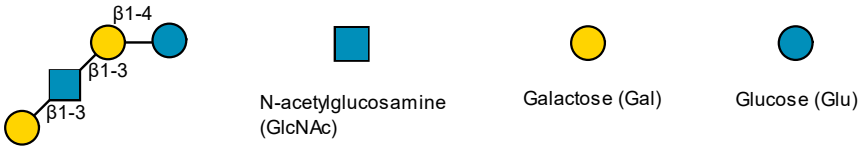
2.1 Identity

Common Name:	Lacto-*N*-tetraose
Abbreviation:	LNT
International Union of Pure and Applied Chemistry (IUPAC) Name:	beta-D-galacto-hexopyranosyl-(1->3)-2-acetamido-2-deoxy-beta-D-gluco-hexopyranosyl-(1->3)-beta-D-galacto-hexopyranosyl-(1->4)-D-gluco-hexopyranose
Chemical Abstracts Service (CAS) Number:	14116-68-8
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

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

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

The identity of Inbiose's LNT has been confirmed by nuclear magnetic resonance (NMR), by comparison with a LNT reference standard (Batch ID: 45/01, IsoSep AB, Sweden) derived from human milk. Based on NMR, the Inbiose LNT 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 LNT 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, 2020b,c). 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 many industrial applications, including the production of specialty substances L-aspartic, inosinic, and adenylic acids, which the human body produces, and U.S. FDA-approved human drugs such as insulin and somatostatin.

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

2.2.1.2 Production Strain

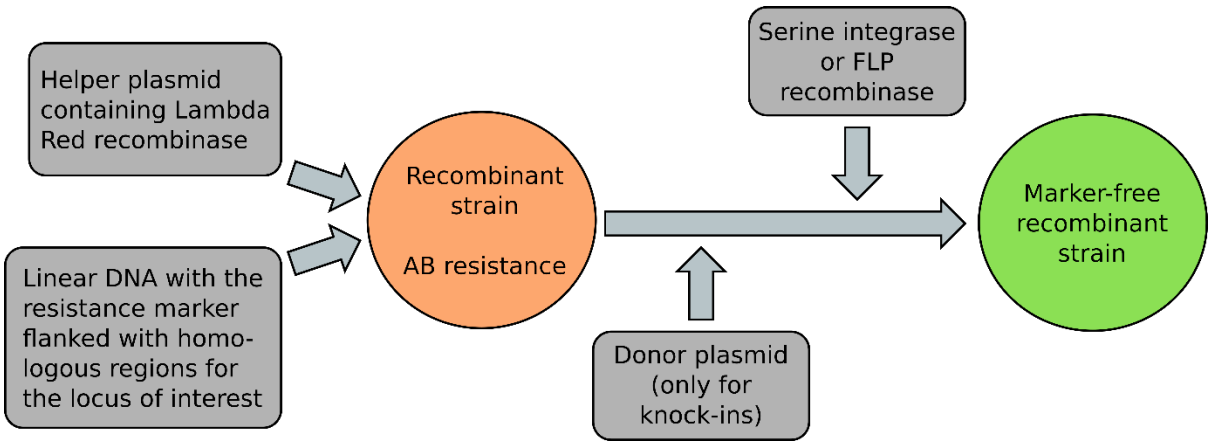
Several modifications, like gene knock-outs, gene insertions and the addition of a production plasmid, were performed on *E. coli* K-12 strain MG1655 to create a LNT production strain. A production strain, INB-LNT_01, has been developed, through which the safety was assessed.

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 inserted into the genome. The removal of the helper plasmid is also validated by (i) PCR and (ii) replica plating on a plate containing the antibiotic for which the marker is present on the helper plasmid. In the case of the PCR test, no amplification was observed when the plasmid is not present; in the case of the replica plate, no growth was 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, need to be added.

All heterologous genes introduced into INB-LNT_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 bio-informatic tools. Also, before and after introducing these heterologous genes into the genome of the production host organism, a full Sanger sequencing of the transcription units was performed to ensure their identity.

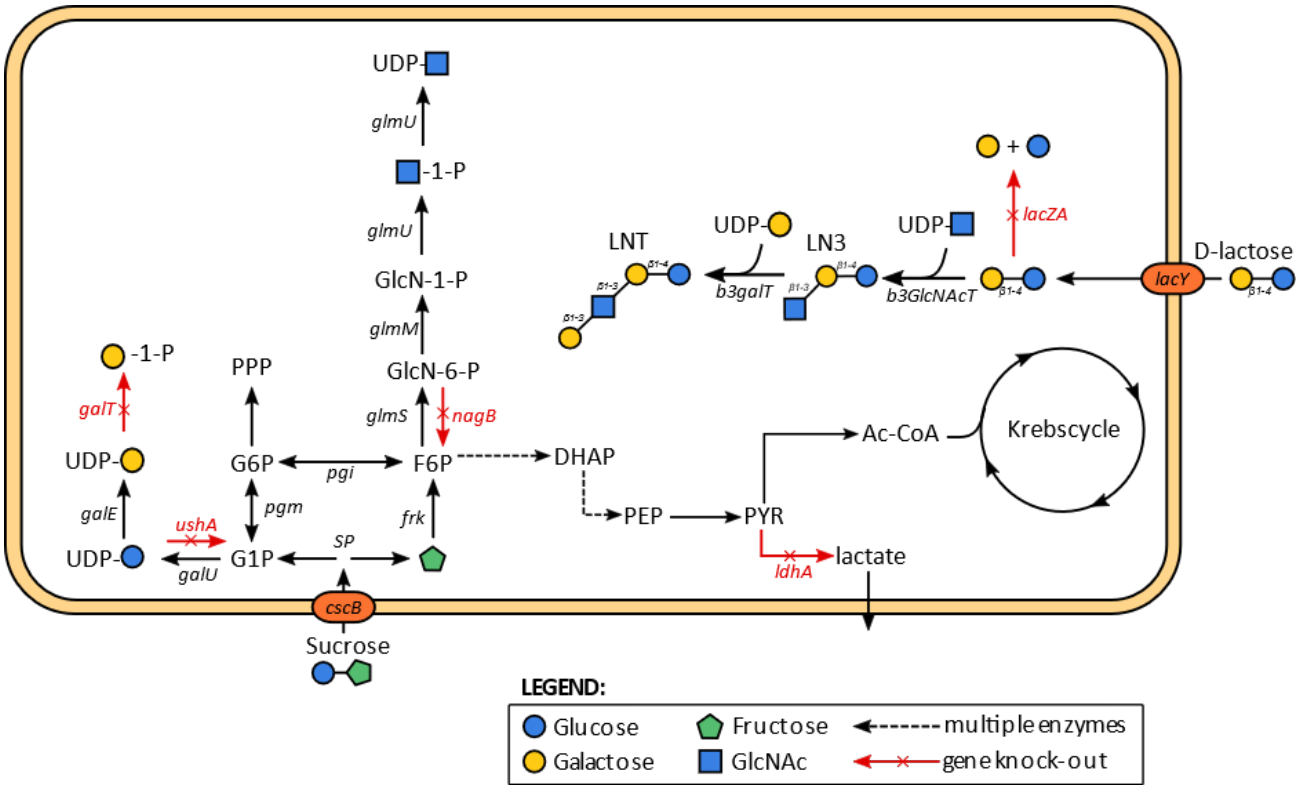
The host organism *E. coli* K-12 strain MG1655 was modified by genomic knock-outs and knock-ins by using the methods, as described above, to obtain efficient biosynthesis of LNT (see Table 2.2.1.2-1 and Figure 2.2.1.2-2).

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
Pseudogulbenkiania ferrooxidans	beta-galactosyltransferase
Salmonella enterica	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 LNT by the introduction of genes throughout the genome (see Table 2.2.1.2-1). In addition to the chromosomal modifications, a plasmid was also introduced in the production host INB-LNT_01 for overexpression of a *Salmonella enterica* beta-galactosyltransferase gene. No antibiotic resistance genes were present on the plasmid. The whole vector was synthesized *de novo* and is named pINB-LNT_01. After strain construction, colony PCR, Sanger sequencing, and WGS checks were performed to verify all genetic modifications introduced in the LNT production strain. Production strain INB-LNT_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 LNT Biosynthetic Pathway in INB-LNT_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; LNT = lacto-*N*-tetraose; PEP = phosphoenolpyruvate; PPP = pentose phosphate pathway; PYR = pyruvate; UDP = uridine diphosphate.

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

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

Production strain INB-LNT_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 LNT with INB-LNT_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 LNT, which is partly secreted into the medium. Afterwards, the remaining intracellular LNT 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 LNT biosynthesis and cannot be found in the final product.

The production strain INB-LNT_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-LNT_01 with deposition number LMBP 12730 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

LNT 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 LNT 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, with 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 LNT Manufacturing Process

In summary, the manufacturing method for LNT entails a fermentation process with a K-12 based production host (see Section 2.2.1) that produces LNT. This host produces LNT through the utilization of a carbon source (sucrose), combined with lactose in a minimal medium. The product is released into the medium. The remaining intracellular LNT 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 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 salts present in the medium are largely removed, which are cations (*e.g.*, magnesium, calcium, ammonium) and anions (*e.g.*, phosphate and sulfate, which are minerals used for growth of the microorganism). 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

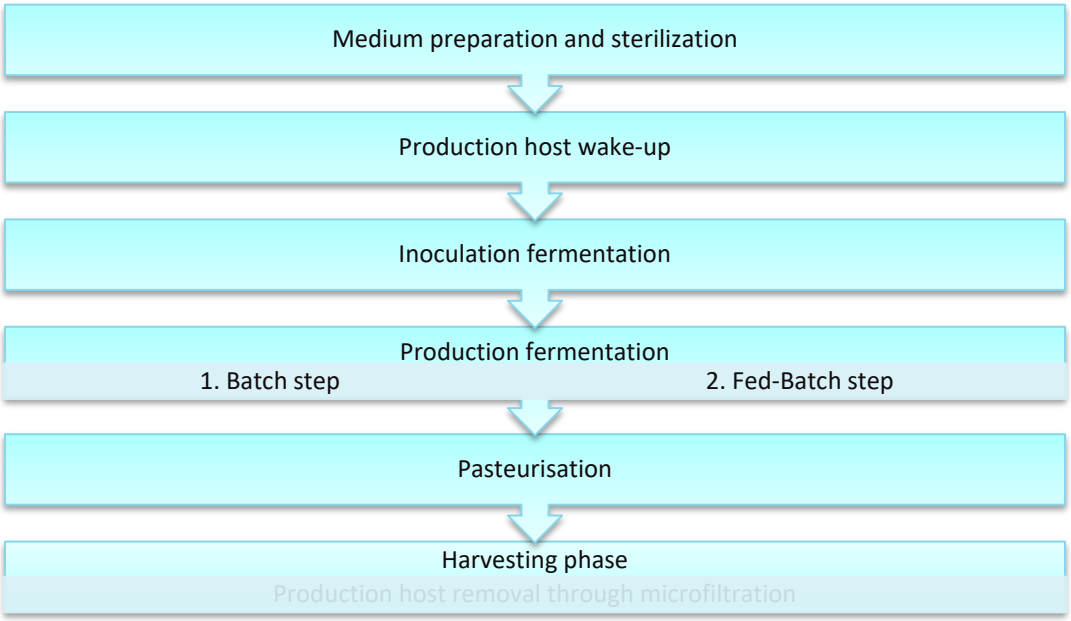
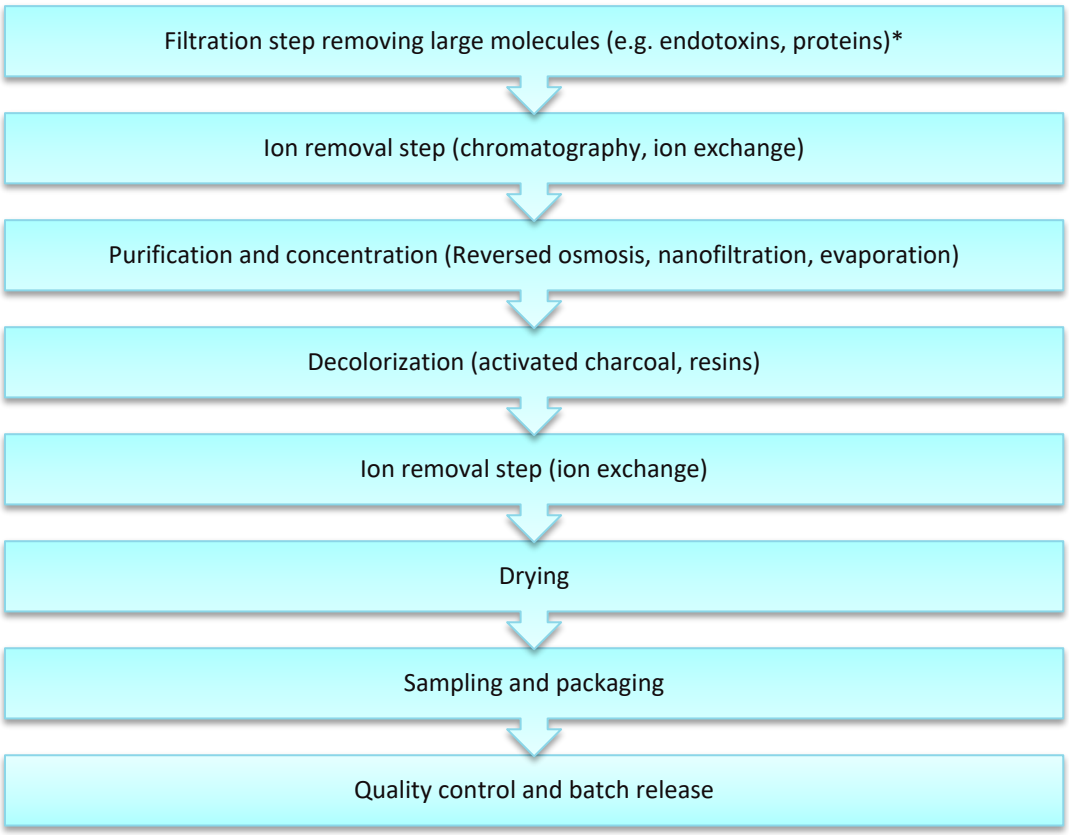


Figure 2.2.3-2 Purification Process



* The filtrations steps are done with cut-offs of 0.1 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 LNT, which includes the amount of LNT and other main carbohydrates, chemical parameters, heavy metals, microbial contaminants, and absence of the genetically modified production strain and endotoxins. The specifications proposed for LNT are presented in Table 2.3.1-1. The specifications of Glycom A/S's (Glycom's) and Jennewein Biotechnologie GmbH's (Jennewein's) LNT preparations (GRN 833 and 923, respectively) are included in the table for comparison (Glycom A/S, 2019; U.S. FDA, 2019, 2021; Jennewein Biotechnology GmbH, 2021). All parameters were determined using compendial or validated methods.

Table 2.3.1-1 Product Specifications for Inbiose's LNT in Comparison to those of the LNT Ingredients in GRN 833 and 923

Parameter	Specification for Inbiose's LNT	Method of Analysis Employed by Inbiose	Specifications Reported for Other LNT Products	
			Glycom's LNT (GRN 833) (U.S. FDA, 2019)	Jennewein's LNT (GRN 923) (U.S. FDA, 2021)
Identification				
Appearance (color)	White	Visual	White to off-white	White to ivory-colored
Appearance (form)	Dry powder	Visual	Powder or agglomerates	Spray-dried powder
Appearance in solution	Clear, colorless to slightly yellow	Visual	NS	NS
Identity (LNT)	Conform to reference standard, LNT derived from human milk	UPLC-RI	RT of standard ± 3%	NS
pH	4.0 to 6.5 (20°C, 10% solution)	Eurofins' internal method, potentiometry	4.0 to 6.0 (20°C, 5% solution)	NS
Carbohydrates, %DM				
LNT	NLT 80.0%	UPLC-RI	NLT 70.0%	NLT 75% DW
Lacto-*N*-triose	NMT 5.0%	UPLC-RI	NMT 10.0 w/w %	NMT 5% (%area)
Lactose	NMT 10.0%	UPLC-RI	NMT 12.0 w/w %	NMT 5% (%area)
para-lacto-*N*-hexaose	NMT 3.5%	UPLC-RI	NMT 3.5 w/w %	NMT 5% (%area)
LNT-fructose	NMT 1.0%	UPLC-RI	NMT 1.0 w/w %	NS
Glucose/galactose	NS	-	NS	NMT 5% (%area)
Sum of human identical milk saccharides[a]	NLT 90.0%	UPLC-RI	NLT 90.0% w/w	NS
Other carbohydrates[b]	NMT 5.0% (%area)	UPLC-RI	NMT 5.0 w/w %	NMT 25% (%area)
Chemical Analysis				
Water content	NMT 9.0%	Karl-Fisher, volumetric	NMT 6.0 w/w %	NMT 9% (w/w)
Protein content	NMT 100 µg/g	Roti®-Nanoquant	NMT 0.01 w/w %	NMT 100 µg/g
Ash content	NMT 0.1% w/w	NEN 6810 (500–550°C)	NMT 0.5 w/w %	NMT 1% (w/w)
Residual endotoxins	NMT 10 EU/g	Ph. Eur. 2.6.14	NMT 10 EU/g	NMT 10 EU/g
Aflatoxin M1	NMT 0.25 µg/kg	LC-MS/MS	NS	NMT 0.25 µg/kg
Heavy Metals				
Arsenic	NMT 0.2 mg/kg	ICP-MS	NS	NMT 0.2 mg/kg
Cadmium	NMT 0.1 mg/kg	ICP-MS	NS	NMT 0.1 mg/kg
Lead	NMT 0.02 mg/kg	ICP-MS	<0.1	NMT 0.02 mg/kg
Mercury	NMT 0.5 mg/kg	ICP-MS	NS	NMT 0.5 mg/kg
Microbiological Contaminants				
Total plate count	NMT 10,000 CFU/g	ISO 4833	NMT 500 CFU/g	NMT 10,000 CFU/g
Enterobacteriaceae	Absent in 10 g	ISO 21528-2	Absent in 10 g	NMT 10 CFU/g
Salmonella spp.	Absent in 25 g	ISO 6579-1	Absent in 25 g	Absent in 25 g
Cronobacter (Enterobacter) sakazakii	Absent in 25 g	ISO/TS 22964	Absent in 10 g	Absent in 10 g

Table 2.3.1-1 Product Specifications for Inbiose's LNT in Comparison to those of the LNT Ingredients in GRN 833 and 923

Parameter	Specification for Inbiose's LNT	Method of Analysis Employed by Inbiose	Specifications Reported for Other LNT Products	
			Glycom's LNT (GRN 833) (U.S. FDA, 2019)	Jennewein's LNT (GRN 923) (U.S. FDA, 2021)
Listeria monocytogenes	Absent in 25 g	AFNOR EGS 38/05-03/17	Absent in 25 g	NS
Bacillus cereus	NMT 50 CFU/g	ISO 7932	NMT 50 CFU/g	NS
Yeasts[c]	NMT 100 CFU/g	ISO 7954	NMT 10 CFU/g	NMT 100 CFU/g
Molds[c]	NMT 100 CFU/g	ISO 7954	NMT 10 CFU/g	NMT 100 CFU/g

CFU = colony forming units; DM = dry matter; DW = dry weight; EU = endotoxin unit; Glycom = Glycom A/S; GRN = GRAS Notice; ICP-MS = inductively coupled plasma mass spectrometry; ISO = International Organization for Standardization; Jennewein = Jennewein Biotechnologie GmbH; LC-MS/MS = liquid chromatography tandem mass spectrometry; LNT = lacto-*N*-tetraose; NLT = not less than; NMT = not more than; NS = not specified; Ph. Eur. = European Pharmacopoeia; RT = retention time; UPLC-RI = ultra-high performance liquid chromatography coupled with refractive index detector.

[a] Human identical milk saccharides is defined as lacto-*N*-triose, lactose, and lacto-*N*-tetraose.

[b] Expressed in area %.

[c] Specification for yeast and mold is combined.

2.3.2 Batch Analysis

Results for the analyses of 5 non-consecutive batches of LNT 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 LNT

Parameter	Specification	Lot Nos.				
		ilex14F03	ilex14F04	ilex14F05	ilex14F10	ilex14F11
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 whitish	Clear, colorless to slightly whitish	Clear, colorless to slightly whitish	Clear, colorless to slightly whitish	Clear, colorless to slightly whitish	Clear, colorless to slightly whitish
pH (20°C, 10% solution)	4.0 to 6.5	6.07	5.66	6.19	5.93	5.20
Carbohydrates, %DM						
LNT	≥80.0%	92.03	92.70	94.11	93.89	91.92
Lacto-*N*-triose	≤5.0%	1.66	2.64	1.58	1.75	2.03
Lactose	≤10.0%	1.87	1.81	0.59	0.93	2.72
para-lacto-*N*-hexaose	≤3.5%	0.85	0.78	0.74	1.49	1.36
LNT-fructose	≤1.0%	0.66	0.27	0.29	0.12	0.22
Sum of human identical milk saccharides[a]	≥90.0%	95.56	97.15	96.28	96.57	96.67
Other carbohydrates	≤5.0%	2.93	1.80	2.68	1.98	1.75
Chemical Analysis						
Water content, volumetric (w/w%)	≤9.0%	5.3	5.6	5.4	4.34	4.8
Protein content (µg/g)	≤100	<25	<25	<25	<25	<25
Ash content (%)	≤0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Residual endotoxins (IU/g)	≤10	<5	<5	<5	<5	<5
Aflatoxin M1 (µg/kg)	≤0.25	<0.01	<0.01	<0.01	<0.01	<0.01
Heavy Metals						
Arsenic (mg/kg)	≤0.2	<0.01	<0.01	<0.01	<0.01	<0.01
Cadmium (mg/kg)	≤0.1	<0.002	<0.002	<0.005	<0.005	<0.005
Lead (mg/kg)	≤0.02	<0.004	<0.004	<0.01	<0.01	<0.01
Mercury (mg/kg)	≤0.5	<0.002	<0.002	<0.01	<0.01	<0.01
Microbiological Contaminants						
Total plate count (CFU/g)	≤10,000	200[b]	200[b]	<100	<100	<100
Enterobacteriaceae (CFU/g)	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

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

Parameter	Specification	Lot Nos.				
		ilex14F03	**ilex14F04**	**ilex14F05**	**ilex14F10**	**ilex14F11**
Listeria monocytogenes	Absent in 25 g	Absent	Absent	Absent	Absent	Absent
Bacillus cereus	≤50	<10	<10	<10	<10	<10
Yeasts (CFU/g)	≤100	<10	<10	<10	<10	<10
Molds (CFU/g)	≤100	<10	<10	<10	<10	<10

CFU = colony forming units; DM = dry matter; IU = International units; LNT = lacto-*N*-tetraose.
[a] Human identical milk saccharides is defined as lacto-*N*-triose, lactose, and lacto-*N*-tetraose.
[b] Estimated value.

2.3.3 Microbiological Endotoxins and Residual Protein Analysis

The content of endotoxins and residual proteins in the LNT 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 LNT 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 LNT product of 5 regulatory batches of INB-LNT_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* on the genome and a subsequence of the beta-galactosyltransferase gene of *Salmonella enterica* and thyA gene of *E. coli* on the plasmid pINB-LNT_01 were targeted to check for residual DNA in the LNT 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 LNT showed no detectable levels of residual DNA in the final product. The limit of detection for the sucrose phosphorylase gene subsequence and the beta-galactosyltransferase and thyA gene subsequence were both below the threshold limit of detection of 10 ng DNA per gram LNT as it is stated in the EFSA guidelines (EFSA, 2018).

2.4 Stability

The stability of Inbiose's LNT is supported by the real-time and accelerated stability studies summarized in GRN 833 and 923. The compositional similarities between Inbiose's LNT and the LNT ingredients summarized in GRN 833 and 923 (see Section 2.3.1) indicate that the stability of the ingredients will be similar. A summary of the real-time and accelerated stability studies, as described in GRN 833 and 923 is provided below.

As described in GRN 833 (U.S. FDA, 2019), the chemical, physical, microbiological, and sensory stability testing of Glycom's LNT (produced from fermentation) was assessed in an ongoing 5-year study under real-time conditions [25°C, 60% relative humidity (RH)]. The 24-month interim results of 2 representative batches confirmed that LNT is stable when stored at ambient room temperatures. The results of an accelerated stability study (40°C, 75% RH) also indicated no changes in the evaluated chemical (LNT, lactose, lactulose, lacto-*N*-triose II, *para*-LNH2, LNT fructose isomer, and water content), physical (appearance and color), and microbiological parameters [aerobic mesophilic total plate count, Enterobacteriaceae, *Salmonella* spp., *Cronobacter (Enterobacter) sakazakii*, *Listeria monocytogenes*, *Bacillus cereus*, yeasts, and molds] in 2 representative batches following storage for up to 24 months. The Arrhenius equation (Peleg *et al*., 2012) was used to extrapolate the results of the accelerated stability study to conclude that the ingredient was stable for at least 5 years when protected from light and stored at room temperature and ambient humidity.

Further to this, Glycom's LNT was subject to stress/forced stability conditions. The LNT product was tested in slightly acidic (pH 4.5) or neutral (pH 6.8) aqueous solutions at 60°C for 8 weeks or 80°C for 4 weeks of storage. In the crystalline and amorphous form, LNT was found to undergo "*minor isomerisation to the LNT fructose isomer*", at neutral pH. Amorphous LNT was "*hydrolysed to glucose and lacto-N-triose II*" under slightly acidic conditions. Hence, the optimal stability of LNT powder was observed to be between pH 5 and 6.

The stability of Jennewein's LNT was assessed in a HMO mixture containing approximately 23% LNT, stored in high-density polyethylene bottles under ambient (25°C, 60% RH) and accelerated (40°C, 75% RH) conditions for 52 and 26 weeks, respectively (GRN 923 – U.S. FDA, 2021). An 8% reduction in LNT content was found at week 26 under ambient conditions; however, as this was not seen at 56 weeks, this was attributed to analytical variability. Moisture increased from 5.7 to 7.8%. Under accelerated conditions, the LNT content decreased, and moisture content increased. These data combined with the data provided in GRN 833 supported the 2-year shelf life of Jennewein's LNT when stored under ambient conditions (U.S. FDA, 2019).

The stability of LNT was also assessed under the intended conditions of use. The LNT ingredient produced by Glycom was assessed in powdered infant formula, as described in Section 2.4.2 of GRN 833 (U.S. FDA, 2019). Glycom's LNT was added to a powdered infant formula supplemented with other HiMOs, long chain polyunsaturated fatty acids (LC-PUFA), vitamins, and minerals was also found to be stable at various temperatures (4°C, 20°C, 30°C, and 37°C) for up to 12 months. Further stability testing in various food matrices (*e.g.*, yoghurts, ready-to-drink flavored milk, and citrus fruit beverages) was conducted with LNnT, a constitutional isomer of LNT. Details of these studies, presented in Section II.D.2 of GRN 547, are incorporated by reference and briefly summarized below (U.S. FDA, 2015). LNnT was found to be stable in these products that were subjected to standard processing *(i.e.*, pasteurization and/or ultra-high temperature heating) and storage conditions (*e.g.*, temperature and shelf-life).

These results show that LNT is anticipated to be stable in most food matrices.

Part 3. § 170.235 Dietary Exposure

3.1 Estimated Intake of LNT

3.1.1 Methods

Inbiose's LNT is intended for use as a food ingredient in term infant formula (0 to 12 months) at concentrations up to 0.8 g/L and toddler formula and other drinks for young children at concentrations up to 0.6 g/L and products other than beverages (*e.g*., baby foods) up to 5 g/kg. These intended use levels are based on the levels of LNT detected in human and bovine milk, as described in GRN 833 and 923. Inbiose's LNT will also be targeted to the general U.S. population in food and beverage products including beverage and beverage bases up to 2.0 g/L, grain products and pastas up to 20.0 g/kg, milk (whole and skim) up to 1.0 g/L and milk products up to 2.0 g/L or 10 g/kg, as described in GRN 833 (see Table 1.3-1). As food uses of LNT are fully substitutional to current GRAS uses previously determined to be GRAS in GRN 833, no changes in dietary intake of LNT are expected from the introduction of Inbiose's LNT ingredient to the U.S. marketplace. A summary of the estimated dietary intake of LNT from food uses described in GRN 833 are presented below and are considered applicable to GRAS uses of LNT described herein.

3.1.2 Intake Estimates for LNT

As described in GRN 833, the estimated intake of LNT as an ingredient in term infant formula (0 to 12 months), toddler formula and other food and beverage products has been estimated from dietary survey data. The intake of LNT described in GRN 833 was estimated using food categories representative of each proposed food use chosen from the National Center for Health Statistics' 2013-2014 National Health and Nutrition Examination Survey (NHANES) (CDC, 2015, 2016; USDA, 2016). Based on the proposed uses, more than 92.2% of the evaluated population groups consisted of eligible LNT consumers, with children at 99.0% representing the greatest proportion of potential consumers. Male adults were established to represent the highest mean and 90th percentile consumer-only intakes of LNT on an absolute basis, at 0.94 and 2.02 g/person/day, respectively. The summary of the estimated dietary intake of LNT in the U.S. population, as described in GRN 833, is provided in Table 3.1.2-1 (U.S. FDA, 2019).

Table 3.1.2-1 Summary of the Estimated Daily Intake of LNT from Proposed Food Uses in the U.S. by Population Group (2013-2014 NHANES Data)*[a]

Population Group	Age Group (Years)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Toddlers	1 to 3	0.89	1.86	98.5	465	0.90	1.88
Children	4 to 10	0.73	1.46	99.0	986	0.74	1.47
Female Teenagers	11 to 18	0.66	1.37	94.5	572	0.70	1.37
Male Teenagers	11 to 18	0.91	1.62	98.2	570	0.93	1.66
Female Adults of Childbearing Age	19 to 40	0.72	1.56	92.9	826	0.78	1.57
Female Adults	19 to 64	0.73	1.69	92.9	1,764	0.79	1.72
Male Adults	19 to 64	0.87	1.97	92.7	1,522	0.94	2.02
Elderly	65 and up	0.61	1.49	92.2	917	0.66	1.53
Total Population	All ages	0.78	1.72	93.8	7,088	0.83	1.77

Table 3.1.2-1 Summary of the Estimated Daily Intake of LNT from Proposed Food Uses in the U.S. by Population Group (2013-2014 NHANES Data)*[a]

Population Group	Age Group (Years)	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile

GRN = GRAS Notice; LNT = lacto-*N*-tetraose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

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

[a] Table adapted from GRN 833 (U.S. FDA, 2019), full intake assessment reported in GRN 833 GRAS determination.

Following the submission of GRN 833, the U.S. FDA requested further exposure estimates for infants aged 0 to 12 months. The mean and 90th percentile consumer-only intakes of LNT among infants (0 to 12 months), on an absolute basis, are provided in Table 3.1.2-2 (GRN 833; U.S. FDA, 2019).

Table 3.1.2-2 Summary of the Estimated Daily Intake of LNT from Proposed Food Uses in the U.S. by Population Group (2013-2014 NHANES Data)*[a]

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

GRN = GRAS Notice; LNT = lacto-*N*-tetraose; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

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

[a] Additional table submitted with GRN 833, following FDA questions (U.S. FDA, 2019). Full intake assessment reported in GRN 833 GRAS determination.

Part 4. § 170.240 Self-Limiting Levels of Use

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

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

Not applicable.

Part 6. § 170.250 Narrative and Safety Information

6.1 Introduction

The first GRAS conclusion notified to the U.S. FDA for LNT was submitted by Glycom in 2018 (GRN 833; U.S. FDA, 2019). A critical and comprehensive review of the publicly available data and information pertaining to the safety of LNT for use as an ingredient in non-exempt infant formula, and various food and beverage products across multiple categories was presented in this notice, and the published information pertinent to the safety of LNT presented by Glycom has served as the basis for subsequent GRAS conclusions for a similar LNT preparation (GRN 923; U.S. FDA, 2021). These LNT preparations are produced *via* microbial fermentation using genetically modified strains of *E. coli* K-12 DH1 or *E. coli* BL21 DE3. Despite differences in manufacturing process, these LNT 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 LNT 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 an opinion supporting the safe use of LNT as an ingredient in a variety of foods, including infant and follow-on formula (EFSA, 2019).

As reported in Section III, Part B of GRN 923, concentrations of LNT in human milk can range from 0.003 to 6.7 g/L, varying depending on ethnicity, Secretor and Lewis-blood type, lactation period, and term vs preterm birth. A thorough detailing of these variations in LNT concentration were reported in Table 7 of Section III Part B in GRN 923 based on geographical location and lactation time (Chaturvedi et al. 1997; Sumiyoshi et al., 2003; Sjögren et al., 2007; Leo et al. 2010; Marx et al., 2014; Alderete et al., 2015; Spevacek et al., 2015; Austin et al., 2016, 2019; Kunz et al., 2017; McGuire et al., 2017; Sprenger et al., 2017; Thurl et al., 2017; Williams et al., 2017; Azad et al., 2018; Ma et al., 2018; Nijman et al., 2018; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019). As such, the use of LNT as an ingredient in non-exempt term infant formula at levels up to 0.8 g/L is within the range that infants are exposed to following the ingestion of human milk.

Based on the equivalence of Inbiose's LNT to other LNT with GRAS status, publicly available data and information establishing the GRAS status of LNT (U.S. FDA, 2019) are therefore incorporated by reference to a previous GRAS evaluation 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 LNT 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 LNT and any newly identified studies relevant to the safety of Inbiose's LNT are provided below.

6.2 Absorption, Distribution, Metabolism, and Excretion

As discussed previously, LNT produced by microbial fermentation is structurally identical to the LNT 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 LNT, has been previously described in detail (U.S. FDA, 2019, 2021). Briefly, HMOs are resistant to enzymatic hydrolysis and are therefore not significantly digested in the upper gastrointestinal tract (Brand-Miller *et al*., 1998; Engfer *et al*., 2000; Rudloff and Kunz, 2012; EFSA, 2019). Once in the large intestine, LNT 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).

6.3 Toxicological Studies

6.3.1 Toxicology Studies Conducted with Inbiose's LNT

Toxicology studies characterizing the genotoxicity and subchronic toxicity of LNT in neonatal rats are presented, as conducted, and provide information on the safety of Inbiose's ingredient. Findings from these studies are consistent with observations previously reported in the published literature and described in GRN 833 and 923: LNT 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 LNT was evaluated in a bacterial reverse mutation assay conducted in accordance with OECD Test Guideline 471 (OECD, 1997) (Tóth-Gönczöl, 2021 [unpublished]). The study included a Preliminary Compatibility Test, a Preliminary Range Finding Test (Plate Incorporation Method), and 2 main assays (Assay 1 – Plate Incorporation Method; Assay 2 – Plate incorporation method without metabolic activation and Pre-Incubation Method with metabolic activation). Concentration of test formulations were determined analytically.

In the Preliminary Compatibility Test, the solubility of LNT was examined using water for injection. LNT was soluble at 100 mg/mL concentration using water for injection (clear solutions).

For the range finding test, *Salmonella* Typhimurium (*S.* Typhimurium) TA98 and TA100 tester strains and *E. coli* strain WP2 *uvrA* were exposed to LNT 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 LNT 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 LNT and as the negative control. Strain-specific positive controls were also included in the presence (2-aminoanthracene for all strains) and absence [9-aminoacridine (TA1537), sodium azide (TA100 and TA1535), 4-nitro-*o*-phenylenediamine (TA98), and methyl-methanesulfonate (WP2 *uvrA*)] 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 *In Vitro* 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 (2µL/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. The mean number of revertant colonies did not show any biologically relevant increase compared to the solvent controls. There were no reproducible dose-related trends and there was no indication of any treatment-related effect. No precipitation nor growth inhibition and no cytotoxic effects of LNT were observed in the main assays in all examined bacterial strains. Only a slightly reduced background lawn was noted in *S.* Typhimurium TA98 and TA1537 with metabolic activation at 5,000 µg/plate concentration in Assay 2.

The mean values of revertant colonies of the negative (vehicle/solvent) control plates were within the historical control range. The positive controls showed a distinct increase of induced revertant colonies in each strain with and without metabolic activation. The viability of the bacterial cells was checked by a plating experiment in each test, and the study was therefore considered to be valid. Under the experimental conditions applied in this study, LNT 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 LNT 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 *In Vitro* Bacterial Reverse Mutation Assay

Concentration (µg/plate)	Revertant Colonies per Plate (Mean ± SD)									
	Without Metabolic Activation (-S9 mix)					With Metabolic Activation (+S9 mix)				
	Salmonella Typhimurium				*Escherichia coli*	*Salmonella* Typhimurium				*Escherichia coli*
	TA98	TA100	TA1535	TA1537	WP2unA	TA98	TA100	TA1535	TA1537	WP2unA
Plate Incorporation Method – Assay 1 (Mean ± SD)										
Water for injection	17.0±1.00	119.7±1.15	13.7±0.58	12.7±0.58	48.7±1.53	23.7±3.79	123.7±4.93	13.7±0.58	13.0±0.00	50.7±2.08
5,000	18.0±2.00	115.7±7.77	13.7±1.53	13.3±0.58	48.7±0.58	23.0±2.65	124.7±4.73	13.3±0.58	13.3±0.58	51.3±0.58
2,500	19.7±2.08	117.3±2.89	15.3±1.15	12.0±1.00	50.0±2.65	23.7±4.62	118.7±2.52	13.0±1.73	13.3±0.58	48.3±0.58
1,250	19.3±3.06	117.7±5.51	12.7±1.53	12.3±0.58	49.0±1.73	23.7±3.79	124.7±3.79	11.3±2.08	13.0±0.00	53.7±1.53
625	18.3±1.53	119.0±2.00	14.0±0.00	14.7±0.58	50.7±4.16	24.7±3.06	120.3±6.51	13.3±1.15	14.3±0.58	49.7±4.73
312.5	16.7±1.15	115.3±2.52	14.0±0.00	12.7±0.58	51.3±2.52	27.7±3.06	121.3±9.07	12.0±1.00	13.7±0.58	52.0±2.00
15.81	18.0±0.00	108.0±5.29	13.0±1.00	11.0±1.00	50.0±1.73	29.0±0.00	122.3±3.21	12.7±3.06	14.7±0.58	55.3±1.15
Positive control#	412.0±22.27	1206.7±16.17	1189.3±20.13	414.7±12.22	1064.0±32.00	2449.3±42.39	2440.0±24.98	214.0±12.00	214.0±9.54	247.3±9.02
Plate Incorporation Method (-S9 mix), Pre-Incubation Method (+S9 mix) – Assay 2 (Mean ± SD)										
Water for injection	18.3±1.53	95.7±1.53	14.3±1.53	13.0±1.73	45.0 ±1.00	21.3±0.58	99.3±3.51	13.7±0.58	12.3±1.53	52.0±1.00
5,000	17.3±1.53	96.3±5.86	15.0±2.00	12.7±2.31	44.7±0.58	22.0±2.00 SR	97.3±1.53	13.0±1.00	12.3±2.08 SR	50.7±1.53
1,581	17.3±1.15	99.7±2.52	14.3±0.58	12.7±1.53	45.0±2.65	21.0±1.00	98.7±1.53	13.3±0.58	13.7±0.58	48.3±0.58
500	16.0±1.00	96.3±3.51	14.7±0.58	13.3±1.15	45.7±0.58	21.7±0.58	102.7±1.53	15.3±1.53	13.7±0.58	51.0±2.65
158.1	17.7±1.15	93.0±4.00	15.7±0.58	11.7±3.21	45.7 ±2.08	21.7±2.08	96.7±4.04	14.3±1.53	11.3±0.58	49.7±2.08
50	17.7±1.15	96.3±3.51	15.0±1.00	12.7±1.15	46.0 ±1.73	20.7±1.53	92.0±3.00	14.7±1.15	12.7±1.53	53.3±0.58
15.81	18.7±2.08	90.7±3.51	15.7±0.58	12.7±1.53	44.0 ±2.00	22.3±2.08	97.7±3.21	16.0±1.00	13.0±1.73	49.0±1.00
Positive control#	503.7±27.6	39.±17.7	85.7±10.3	502.3±40.0	971.3±66.5	112.0±6.6	59.7±14.6	1189.3±38.2	921.7±63.6	145.3±26.4

S9 = metabolic activation; SD = standard deviation.
# List of positive controls is included in Table 6.3.1.1-1

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

The potential clastogenicity and aneugenicity of LNT was evaluated in an *in vitro* micronucleus test with human peripheral blood lymphocytes. This study was conducted in accordance with OECD Test Guideline 487 (OECD, 2016) (Buskens, 2021 [unpublished]).

For the pulse exposure, the human lymphocytes were exposed to LNT at concentrations of 0 (water for injection and vehicle), 63, 125, 250, 500, 1,000, or 2,000 µg LNT/mL in the presence (positive control: cyclophosphamide) and absence (positive controls: mitomycin C and colchicine) of external metabolic activation (S9 mix) for 3 hours followed by a 24-hour recovery. For the continuous exposure, the human lymphocytes were exposed to LNT at concentrations of 0 (water for injection and vehicle), 63, 125, 250, 500, 1,000, or 2,000 µg LNT/mL in the absence (positive control: mitomycin C and colchicine) of external metabolic activation (S9 mix) for 24 hours with no recovery.

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

Based on the results of this study, LNT was concluded to have no potential for clastogenicity or aneugenicity in human lymphocytes at doses up to 2,000 µg/mL (the OECD Test Guideline 487 maximum recommended concentration).

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

Concentration (µg/mL)	Cytostasis (%)	Culture	Number of Analyzed Micronucleated Binucleated Cells	Total Micronucleated Binucleated Cells	
				Per Culture	Per Dose
Vehicle	0	C1	1,000	6	10
		C2	1,000	4	
500	6	C1	1,000	1	6
		C2	1,000	5	
1,000	16	C1	1,000	7	11
		C2	1,000	4	
2,000	18	C1	1,000	4	9
		C2	1,000	5	
MMC-C: 0.25 µg/mL	44	C1	1,000	44	87****
		C2	1,000	43	
Colch: 0.1 µg/mL	75	C1	638	11	21****
		C2	578	10	
3-h Treatment, 27-h Harvest Time: With Metabolic Activation (+S9 mix)					
Vehicle	0	C1	1,000	3	4
		C2	1,000	1	
500	-1	C1	1,000	1	3
		C2	1,000	2	
1,000	-4	C1	1,000	3	7
		C2	1,000	4	
2,000	-6	C1	1,000	5	7
		C2	1,000	2	
CPA: 15 µg/mL	59	C1	1,000	29	45***
		C2	1,000	16	

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

Concentration (µg/mL)	Cytostasis (%)	Culture	Number of Analyzed Micronucleated Binucleated Cells	Total Micronucleated Binucleated Cells	
				Per Culture	Per Dose
24-h Treatment, 24-h Harvest Time: Without Metabolic Activation (-S9 mix)					
Vehicle	0	C1	1,000	0	1
		C2	1,000	1	
500	5	C1	1,000	0	1
		C2	1,000	1	
1,000	4	C1	1,000	0	1
		C2	1,000	1	
2,000	4	C1	1,000	0	0
		C2	1,000	0	
MMC-C: 0.15 µg/mL	48	C1	1,000	20	37****
		C2	1,000	17	
Colch: 0.05 µg/mL	99	C1	73	30	59***
		C2	93	29	

C1 = Culture 1; C2 = Culture 2; Colch = Colchicine; CPA = Cyclophosphamide; MMC-C = Mitomycin C.
Vehicle: water for injection.
* Significantly different from control group (Chi-square test), * P < 0.05, ** P < 0.01, *** P < 0.001, or **** P < 0.0001.

6.3.1.2 Acute Oral Toxicity Test (OECD Test Guideline 425)

The median lethal dose (LD_{50}) of LNT was assessed in a single dose acute toxicity study conducted in accordance with OECD Test Guideline 425 (OECD, 2008) (Orosz, 2021 [unpublished]). Three female Crl:WI Wistar rats were administered a single dose of 5,000 mg LNT/kg body weight (bw) dissolved in distilled water *via* gavage, followed by a 14-day observation period. There was no mortality during the study nor test item-related effects were observed; hence, the LD_{50} was concluded to be greater than 5,000 mg LNT/kg bw (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 LNT and select a maximum dose for the subsequent 90-day subchronic toxicity study (Chevalier, 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 3'-SL/kg bw/day by gavage from Post-natal Day (PND) 7 to 27 at 10 mL/kg bw/day. Satellite groups of SD rats (4/sex/group) were also administered 3,000 and 5,000 mg/kg bw/day, to develop and further validate the bioanalytical method of detection for LNT in plasma and urine. All animals were observed daily for mortality and clinical signs. Body weight and food consumption (after weaning) were recorded at designated intervals. Hematology and blood chemistry parameters were measured in principal animals at the end of the treatment period.

On PND 28 (after at least 14 hours fasting), the principal animals were euthanized and a complete macroscopic *post-mortem* examination of the principal thoracic and abdominal organs was performed. Selected organs were weighed. Satellite rats received LNT treatment *via* oral gavage daily till PND 27. Blood samples were collected on PND 22 at designated time-points (*i.e.*, 0, 1, 4, and 6 hours after LNT gavage). 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 LNT. There were 3 unscheduled deaths in the 5,000 mg/kg bw/day group; 2 female rats were found on PND 12 and a satellite female rat on PND 27. No post-mortem macroscopic examinations could be performed as the animals were cannibalized. The satellite female showed hunched posture, piloerection, coldness to the touch, hypoactivity, hard abdomen with increase in size, abdominal breathing, and half-closed eyes before death. One unscheduled death occurred in the 4,000 mg/kg bw/day group when a female rat was found dead on PND 22 with no clinical signs before death. As translucent content was observed in the thoracic cavity at necropsy, the death was considered procedure-related.

No effects on mean body weight, body weight change, and food consumption were observed. Compared with controls there were some statistically significant differences; however, these differences were of minimal amplitude and no dose-response relationship was identified. Therefore, any effects related to the test article were considered unlikely. There were no treatment-related effects on clinical signs, mean hematology or blood biochemistry parameters. When compared with controls, there were a few statistically significant differences in hematology parameters; however, these differences were of minimal amplitude and lacked dose level relationship, some changes were not consistent between both sexes and when compared with the historical control data, they were considered of no biological and toxicological importance. There were no test item treatment-related changes on mean blood biochemistry parameters and there were no relevant findings when compared with historical control data and/or there were but

without biological importance (*e.g*., but not limited to decreased urea concentrations in all LNT treated groups of males and in mid- and high-LNT group of females or decreased liver enzyme activities).

Equivocal increases in adrenal gland weights were noted at all dose levels. Slightly increased mean absolute and relative-to-body adrenal gland weights were noted in both sexes at ≥3,000 mg/kg bw/day. Although changes were poorly dose-related in magnitude, a relationship with the test item administration could not be excluded. These increases in adrenal weights were not considered to be adverse given their low magnitude and absence of macroscopic correlates. Other statistically significant changes were not considered biologically significant as there was no dose-response relationship or statistical significance at higher doses. There were no test item-related macroscopic observations noted at the end of the treatment period. None of the macroscopic findings were considered LNT-related because they were consistent with spontaneously occurring findings described in the literature, the findings were distributed randomly among groups, or their appearance was similar to findings found in controls. A summary of the statistically significant observations in the 21-day dose range finding study using Inbiose's 6´-SL sodium salt ingredient is provided below in Table 6.3.1.3-1.

Therefore, 5,000 mg/kg bw/day, the highest tested dose of LNT in this study, was considered 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 LNT Ingredient

Parameters	Exposure	Sex	Dose Group (LNT mg/kg bw/day)			
			0	3,000	4,000	5,000
Body Weight/Mean Body Weight Change (Mean values ± SD)						
Mean body weight (g) (1)	Day 4	F	24±1.8	24 ±2.0	**26*±1.7**	25±0.6
Mean body weight (g) (1)	Day 8	M	36±1.9	37 ±1.5	38±2.3	**38*±1.4**
Mean body weight (g) (1)	Day 8	F	35±2.1	35±2.0	37±2.0	**38**±1.0**
Mean body weight (g) (1)	Day 11	M	43±1.7	45 ±1.9	**46*±2.9**	**48**±1.3**
Mean body weight (g) (1)	Day 11	F	42±2.2	43±2.3	**45*±2.4**	**49**±1.2**
Mean body weight (g) (1)	Day 15	M	61±2.6	62 ±2.5	62±2.9	**65*±2.3**
Mean body weight (g) (1)	Day 15	F	59±3.2	59±3.8	61±3.3	**65**±2.1**
Mean body weight change (g) (1)	Day 4/8	F	11±0.7	11±1.2	11±0.7	**13**±0.8**
Mean body weight change (g) (3) SD (K)	Day 8/11	M	7±0.5	8±0.9	9±0.9	**10 **±0.4**
Mean body weight change (g) (3) SD (K)	Day 8/11	F	8±0.5	8±1.4	9±0.7	**11**±0.6**
Mean body weight change (g) (1)	Day 11/15	M	18±1.2	17 ±1.4	**16**±1.1**	17±1.1
Mean body weight change (g) (1)	Day 15/18	M	15±1.3	15±2.4	14±1.2	**13**±0.9**
Mean body weight change (g) (1)	Day 18/21	F	18±1.7	18±2.0	**15*±1.1**	17±1.3
Organ Weights (Mean values ± SD)						
Adrenal glands (g) (1) Mean weight	Day 28	M	0.02713±0.004	**0.03325**±0.003**	**0.03213*±0.004**	**0.03288*±0.004**
Adrenal glands (g) (1) Mean % body	Day 28	M	0.03068±0.006	**0.03755**±0.003**	**0.03737**±0.003**	**0.03672*±0.004**
Adrenal glands (g) (1) Mean weight	Day 28	F	0.02400±0.004	**0.02975*±0.004**	**0.03071**±0.005**	**0.03333**±0.002**
Adrenal glands (g) (1) Mean % body	Day 28	F	0.03059±0.006	**0.03799*±0.005**	**0.03690*±0.005**	**0.03903**±0.002**
Epididymides (g) (1) Mean weight	Day 28	M	0.12150±0.010	0.11100±0.011	**0.09638**±0.010**	0.10775±0.019
Epididymides (g) (1) Mean % body	Day 28	M	0.13683±0.013	0.12568±0.015	**0.11255*±0.011**	0.12044±0.021
Testes (g) (1) Mean weight	Day 28	M	0.70988±0.090	0.63750±0.037	**0.62950* ±0.041**	0.66675±0.057
Thymus (g) (1) Mean weight	Day 28	M	0.38488±0.056	0.41075±0.036	**0.31863*±0.041**	0.42550±0.050

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

Parameters	Exposure	Sex	Dose Group (LNT mg/kg bw/day)			
			0	3,000	4,000	5,000
Thymus (g) (1) Mean % body	Day 28	M	0.43181±0.050	0.46423±0.041	**0.37115* ±0.037**	0.47512±0.052
Thyroid glands (g) (1) Mean weight	Day 28	M	0.00813 ±0.002	**0.01163**±0.002**	0.00950±0.002	0.00963±0.002
Thyroid glands (g) Mean % body	Day 28	M	0.00914±0.002	**0.01309**±0.002**	0.01102 ±0.002	0.01078±0.002
Kidneys (g) (1) Mean % body	Day 28	F	1.12±0.048	**1.20**±0.049**	1.09 ±0.036	1.12±0.073
Liver (g) (3) Mean % body	Day 28	F	3.59 ±0.216	3.57±0.312	**3.32$^{\#}$ ±0.118**	**3.31$^{\#}$±0.134**
Hematology (Mean values ± SD)						
RBC (T/L) (1)	Day 22	M	5.32±0.217	**5.69**±0.239**	**5.72**±0.089**	**5.59*±0.221**
HB (G/L) (1)	Day 22	M	12.0±0.63	**12.6*±0.42**	**12.6±0.24***	12.3 ±0.32
PCV (L/L) (3) SD (L)	Day 22	M	0.38±0.018	**0.39$^{\#}$±0.012**	**0.39±0.010***	0.38 ±0.011
PLT (G/L) (3) SD (K)	Day 22	M	1114±119.2	**1267$^{\#}$±106.7**	1171±68.0	1145 ±118.7
RTC % (1)	Day 22	M	14.85±0.839	**13.70 *±0.965**	**12.26**±0.499**	**12.76**±1.048**
RTC (%) (1)	Day 22	F	13.59±2.087	12.58 ±1.651	**10.77**±0.681**	**11.37*±1.175**
RTC (T/L) (1)	Day 22	M	0.79±0.053	0.78±0.053	**0.70**±0.034**	**0.71*±0.063**
RTC (T/L) (1)	Day 22	F	0.78±0.152	0.76±0.100	**0.64*±0.035**	0.67 ±0.072
MCV (fL) (1)	Day 22	F	69.6±1.99	**66.8*±1.90**	67.7±1.39	67.2±2.16
MCHC (pg) (1)	Day 22	F	22.5±1.22	**21.2*±0.88**	21.8±0.58	21.6±0.69
Blood Biochemistry (Mean values ± SD)						
PHOS (mmol/L) (1)	Day 22	M	3.30±0.221	2.93±0.233	3.08±0.427	**2.81*±0.303**
PHOS (mmol/L) (1)	Day 22	F	3.21±0.362	3.00±0.241	2.84±0.157	2.99±0.146
UREA (mmol/L) (1) SD (L)	Day 22	M	5.5±1.37	**4.1*±1.47**	**4.0±0.37***	**3.7**±0.74**
UREA (mmol/L) (1) SD (L)	Day 22	F	5.9±0.94	5.2±1.70	**3.6±1.02****	**4.2*±1.00**
ALP (U/L) (1)	Day 22	M	694±85.1	724±77.1	599±70.7	**579*±95.6**
ASAT (U/L) (1)	Day 22	M	102±8.7	101±8.5	102±8.2	**88**±8.6**

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

Parameters	Exposure	Sex	Dose Group (LNT mg/kg bw/day)			
			0	3,000	4,000	5,000
ASAT (U/L) (1)	Day 22	F	104±16.4	97±8.6	100±7.5	**85**±5.5**
ALAT (U/L) (1)	Day 22	M	58±9.3	48±13.6	**38**±4.4**	**38**±4.5**
ALAT (U/L) (1)	Day 22	F	53±10.0	**37**±6.8**	**41*±6.6**	43±8.2
Na+ (mmol/L) (1)	Day 22	F	143.9±0.58	142.7±1.72	**141.4**±1.08**	143.2±1.50
CL- (mmol/L) (1)	Day 22	F	106.8±1.46	105.6±1.98	**103.8**±2.16**	104.9±0.74

ALAT = alanine aminotransferase; ALP = alkaline phosphatase; ASAT = aspartate aminotransferase; bw = body weight; Cl- = chloride; F = female; HB = hemoglobin; LNT = lacto-*N*-tetraose; M = male; MCHC = mean cell hemoglobin concentration; MCV = mean cell volume; Na+ = sodium; PCV = packed cell volume; PHOS = inorganic phosphorus; PLT = platelets; RBC = erythrocytes; SD = standard deviation; UREA = urea.
* P<0.05, ** P<0.01 (1) DUNNETT TEST
# P<0.05, ## P<0.01 (3) DUNN TEST
(K) KOLMOGOROV-LILLIEFORS TEST P<0.01
(L) LOGARITHMIC TRANSFORMATION Assigned control group(s): 1.

6.3.1.4 Subchronic Toxicity Study (OECD Test Guideline 408)

Currently, a 90-day study is being conducted to evaluate the potential toxic effects of Inbiose's LNT on the development of juvenile rats, following daily oral administration, from PND 7 to at least PND 97 (Bentz, 2021 [unpublished draft report due September 2021]). 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 LNT

Pertinent studies conducted with Inbiose's LNT 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 LNT.

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

Type of Study	Species or Cell Type	Length of Study	LNT Dose and Route of Administration	Result	Reference
Studies Conducted with Inbiose LNT					
Single dose acute toxicity study up and down procedure (OECD TG 425)	Three female Crl:WI Wistar rats	Single oral dose followed by a 14-day observation period	5,000 mg/kg bw LNT dissolved in distilled water – the highest recommended dose	LD_{50} of LNT was found to be greater than 5,000 mg/kg bw.	Orosz (2021) [unpublished]
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 (distilled water, vehicle); up to 5,000 µg/plate (±S9 mix)	LNT is non-mutagenic under the conditions of this test.	Tóth-Gönczöl (2021) [unpublished]
In vitro micronucleus assay (OECD TG 487)	Human peripheral blood lymphocytes	+S9 = 3 h -S9 = 3 and 24 h	0 (water for injection, vehicle) 125, 250, 500, 1,000, and 2,000 µg LNT/mL – the highest recommended dose	LNT did not induce any chromosome damage, or damage to the cell division apparatus.	Buskens (2021) [unpublished]
Preliminary toxicity study by oral route (gavage) in Juvenile rats	Group of 8 male and 8 female juvenile SD rats Satellite group: 4 male and female juvenile SD rats	21 days from PND 7	0 (water for injection, vehicle) 3,000, 4,000, or 5,000 mg/kg bw/day of LNT, by gavage Satellite group: 3,000, or 5,000 mg/kg bw/day by gavage	5,000 mg/kg bw/day was selected as the appropriate highest dose for the main 90-day study.	Chevalier (2021) [unpublished]

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

Type of Study	Species or Cell Type	Length of Study	LNT Dose and Route of Administration	Result	Reference
90-day toxicity study by oral route (gavage) in Juvenile rats followed by a 5-week treatment-free period (OECD TG 408)	Groups of 10 male and 10 female neonatal Crl:CD(SD) rats In addition, 5 males and 5 females in control and 5,000 mg/kg bw/day group for recovery period For toxicokinetic assessment, groups of 3 male and 3 female satellite animals	90 days	0 (water for injection), 1,500, 3,000, or 5,000 mg/kg bw/day of 3'-SL sodium salt, by oral gavage	In progress.	Bentz (draft report due September, 2021) [unpublished]

bw = body weight; *E. coli* = *Escherichia coli*; h = hours; LD_{50} = median lethal dose; LNT = lacto-*N*-tetraose; 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 LNT Ingredients

Pivotal safety data and information has been discussed previously and is hereby incorporated by reference to Section 6.4 of GRN 833 and Section VI Part C of GRN 923 (U.S. FDA, 2019, 2021). Analytical data of Inbiose's LNT product establishes the ingredient as chemically identical to its LNT counterpart in human breast milk (see Section 2.1.1). Based on analytical data presented demonstrating that LNT produced by Inbiose is of equal or greater purity to LNT preparations that have previously been determined to be GRAS, studies characterizing the toxicity and safety of LNT in animal models are considered relevant to the safety assessment of Inbiose's ingredient.

No evidence of toxicity related to the administration of LNT has been reported in any previous LNT GRAS Notice submission (U.S. FDA, 2019, 2021). Additionally, there were no new data identified evaluating the potential toxicological or genotoxic effects of the ingredient since the previous LNT GRAS determination was prepared.

Furthermore, EFSA has issued a scientific opinion on the HiMO as a novel food, which indicates that the no-observed-adverse-effect letter (NOAEL) of LNT is 4,000 mg/kg bw/day in rats, and that there is no concern regarding its genotoxicity at concentrations up to 5,000 µg/plate. The toxicological studies in GRN 833 and 923 are briefly summarized below in Table 6.3.2-1.

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

Type of Study	Species or Cell Type	Length of Study	LNT Dose and Route of administration	Result	Reference
Studies Conducted with Glycom's LNT (GRN 833)					
Bacterial reverse mutation test	*S.* Typhimurium strains TA98, TA100, TA1535, TA1537, and *E. coli* strain WP2 uvrA (pKM101)	Plate incorporation assay and pre-incubation assay	Up to 5,106.1 µg/plate[a] (±S9)	LNT is non-mutagenic at concentrations up to 5,106.1 µg/plate.	Phipps *et al.* (2018)
In vitro mammalian cell micronucleus test	Human lymphocytes	+S9 = 3 h -S9 = 3 and 24 h	Up to 2,042.44 µg/mL[b]	LNT is neither clastogenic nor aneugenic at concentrations up to 2,042.44 µg/mL.	
14-day oral toxicity study	Groups of 8 male and 8 female neonatal rats	14 days	0 (water for irrigation), 3,250, or 4,000 mg/kg bw/day of LNT, by gavage	4,000 mg/kg bw/day of LNT is the highest dose selected for the subchronic study.	
90-day oral toxicity study	Groups of 10 male and 10 female neonatal Crl:CD(SD) rats	90 days	0 (water for irrigation), 1,000, 2,500, or 4,000 mg/kg bw/day LNT, by gavage Reference control: 4,000 mg/kg bw/day OF powder	NOAEL is 4,000 mg/kg bw/day of LNT.	
Studies Conducted with Jennewein's LNT as Part of an HMO Mixture[c] (GRN 923)					
Bacterial reverse mutation assay	*S*. Typhimurium strains TA98, TA100, TA102, TA1535, and TA1537	Plate incorporation test and pre incubation test	5, 10.0, 31.6, 100, 316, or 600 mg of the HMO mixture per plate containing 1.2, 2.4, 7.5, 23.7, 74.9, and 142.2 mg LNT per plate	The HMO mixture, and the LNT contained therein, was not mutagenic under the conditions tested.	Parschat *et al.* (2020)
In vitro micronucleus assay	Human peripheral blood lymphocytes	4 or 24 h (±S9)	7.5, 15, 30, and 60 mg HMO mixture/mL medium (equivalent to 1.8, 3.6, 7.1, and 14.2 mg LNT/mL medium)	The HMO mixture was not genotoxic under the tested conditions at concentrations up to 60 mg/mL (14.2 mg/mL LNT).	
Seven-day pilot dietary toxicity study	Groups of 5 female CD/Crl:CD rats	7 days	A control diet or the same diet containing 10% of an HMO mixture (equivalent to 2.37% LNT)	No HMO-related differences in behavior, appearance and consistency of the feces, bw, bw gain, or feed consumption were observed.	

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

Type of Study	Species or Cell Type	Length of Study	LNT Dose and Route of administration	Result	Reference
90-day feeding study	Groups of 10 male and female CD/Crl:CD rats	90 days	A control diet or the same diet containing 10% of an HMO mixture (equivalent to 2.37% LNT)	NOAEL for this study was 5.67 g/kg bw/day for male rats and 6.97 g/kg bw/day for the female rats. This resulted in a mean intake of LNT of 1.34 g/kg bw/day in males and 1.65 g/kg bw/day in females	
21 day-neonatal piglet study	Groups of 6 male and female LD-2 Domestic Yorkshire Crossbred Swine (farm pig)	21 days	• A control diet; or • Oligosaccharide blend (2.8 g 2'-FL/L, 0.6 g 3-FL/L, 1.2 g LNT/L, 0.2 g 3'-SL/L, and 0.2 g 6'-SL/L) in the diet; or • Oligosaccharide blend (3.9 g 2'-FL/L, 0.8 g 3-FL/L, 1.6 g LNT/L, 0.3 g 3'-SL/L, and 0.3 g 6'-SL/L) in the diet	The Oligosaccharide blend was well tolerated and did not produce adverse effects on the growth and development of the pigs. No Oligosaccharide blend-related mortalities occurred. The clinical pathology values and macroscopic and microscopic findings at necropsy did not reveal a relationship to treatment with the Oligosaccharide blend at the concentrations evaluated. No adverse findings in gross or histopathology were noted.	Hanlon (2020)

2'-FL = 2'-fucosyllactose; 3'-SL = 3'-sialyllactose sodium salt; 3-FL = 3-fucosyllactose; 6'-SL = 6'-sialyllactose sodium salt; bw = body weight; *E. coli* = *Escherichia coli*; Glycom = Glycom A/S; GRN = GRAS Notice; h = hours; Jennewein = Jennewein Biotechnologie GmbH; LNT = lacto-*N*-tetraose; NOAEL = no-observed-adverse-effect level; OECD = Organisation for Economic Co-operation and Development; OF = oligofructose; *S.* Typhimurium = *Salmonella* Typhimurium; S9 = metabolic activation system; SD = Sprague-Dawley.

[a] Due to a minor error in the application of the correction factor, the high concentration slightly exceeded the intended high concentration of 5,000 µg/plate – the OECD 471 guideline maximum recommended concentration.

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

[c] HMO mixture = 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein using fermentation.

6.3.2.1 Summary of Pivotal Repeat Dose Toxicity Study Reported in GRN 833

As the final results of Inbiose's subchronic toxicity study are pending, please find a detailed summary of a subchronic study with an approved LNT ingredient, incorporated by reference to Sections 6.4.1.1.1 and 6.4.1.1.2 of GRN 883 (U.S. FDA, 2019).

The sub-chronic toxicity study using Glycom's LNT ingredient was published by Phipps *et al*. (2018). This study is considered a pivotal safety study that support the safe use of Inbiose's LNT ingredient under the proposed food categories and use levels, as described in the Section 1.3. Both Glycom's and Inbiose's LNT ingredients are produced by fermentation using genetically engineered *E. coli* K-12 strain. In addition, Inbiose's LNT has a comparable carbohydrate content to LNT product manufactured by Glycom.

Briefly, a 14-day repeat dose toxicity study using neonatal Crl:CD(SD) rats was performed to select a suitable high dose for the subsequent sub-chronic toxicity study with Glycom's LNT ingredient and to address any short-term effects, mainly the gastrointestinal intolerance. The neonatal rats (8/group/sex) received 0 (water for irrigation), 3,250 or 4,000 LNT mg/kg bw/day, by oral gavage, once daily from PND 7. No test item-related deaths or clinical signs were reported. One male dosed at 4,000 mg/kg bw/day was found dead on Day 14 of dosing. The death was considered as an isolated incident unrelated to the treatment with LNT. There were no test item-related effects on the final body weights. No test item-related macroscopic abnormalities were observed at necropsy. It was concluded that the dose of 4,000 mg/kg bw/day was a suitable high dose for the 90-day toxicity study.

In the GLP 90-day study conducted according to the OECD Test Guideline 408 (1998), the neonatal Crl:CD(SD) rats (10/group/sex) received 0 (water for irrigation), 1,000, 2,500 or 4,000 LNT mg/kg bw/day, by oral gavage, once daily from the PND 7 for at least 90 days. In addition, after 90-day treatment period, 5 rats/group/sex were included in the recovery phase, during which the rats were kept un-dosed for additional 4 weeks, to assess whether any effects observed at the end of the 90-day treatment period persist, partially or fully recover. For direct comparison against the high LNT dose group, a reference control group (10 rats/sex) received GRAS non-digestible oligosaccharide fructooligosaccharides (FOS) at a dose level of 4,000 mg/kg bw/day to assess any fiber specific effects.

No test item-related deaths, clinical signs, or ophthalmological findings were observed at the end of the treatment period. The 6 reported deaths (1 male and 1 female from the vehicle control group, 1 male and 1 female from low dose group, 1 reference control female and 1 female from the highest dose group), were concluded by the authors as incidental and unrelated to treatment with LNT.

The mean final body weights, mean body weight gain, mean food consumption and the mean organ weights were considered not affected by LNT treatment. The food intake was slightly (approximately 8%), yet statistically significantly lower for the males dosed with LNT at 4,000 mg/kg bw/day when compared with vehicle controls. However, because of the similar mean food intake observed in the reference FOS group of males, this finding was considered to be unrelated to the LNT treatment. No biologically relevant differences in food consumption were observed during the treatment-free period.

The statistically significant increases in testes weights were noted for LNT mid (2,500 mg/kg bw/day) and high (4,000 mg/kg bw/day) dosed males when compared with vehicle controls. These findings lacked dose repose relationship. The statistically significantly increase of kidney, liver, ovary, and spleen weights relative to body weight were only observed after treatment-free period for the LNT high dosed (4,000 mg/kg bw/day) females; thus, these changes were also considered unrelated to LNT treatment. The macroscopic and microscopic findings at the end of the treatment revealed only incidental findings in all groups and were all consistent with spontaneously occurring in the SD rats of this age.

There were no test item-related effects on the pre-weaning development parameters or the sexual maturation (as evaluated by balano-preputial skinfold separation and vaginal opening for males and females, respectively). Similarly, no test item-related differences in measured behavior parameters were

noted. As there were no indications of the interruption of the pituitary-thyroid axis during the study, the thyroid hormones analysis was not performed.

No test item-related differences in blood biochemistry, urinalysis or hematology parameters were detected between LNT-treated groups and the vehicle controls at the end of the 90-day treatment or the recovery phase.

The statistically significant changes in biochemistry parameters at the end of the treatment period included increased alanine aminotransferase (ALT) for LNT dosed males at 2,500 mg/kg bw/day and 4,000 mg/kg bw/day, increased and decreased urea for males dosed with LNT at 2,500 mg/kg bw/day and 4,000 mg/kg bw/day, respectively; increased cholesterol, calcium and phosphorus, and reduced potassium for LNT dosed males at 4,000 mg/kg bw/day/day; decreased and increased aspartate aminotransferase (AST) for females dosed with LNT at 2,500 mg/kg bw/day and 4,000 mg/kg bw/day respectively; reduced creatinine and urea, and increased chlorine for LNT high (4,000 mg/kg bw/day) dosed females. These findings lacked dose-response relationship and/or were limited to 1 sex (*e.g.*, increased phosphorus for the high LNT-dosed males and reduced creatinine for LNT mid and high dosed females). All individual reported values were within the historical control data (HCD) ranges and reflected normal biological variations. No statistically significant differences were observed at the end of the treatment-free period (Phipps *et al.*, 2018).

The statistically significant changes in hematology parameters at the end of the treatment period included: increased mean cell hemoglobin for LNT dosed males at 4,000 mg/kg bw/day and mean cell hemoglobin concentration for both LNT dosed females and males at 4,000 mg/kg bw/day; reductions in red cell distribution width for LNT dosed females at 2,500 mg/kg bw/day and 4,000 mg/kg bw/day, reductions in mean cell volume and hematocrit for only LNT dosed females at 4,000 mg/kg bw/day. Overall, the differences in hematology parameters were minor, limited to 1 sex and the major reported individual values were within the HCD ranges and therefore reflected normal biological variations. The statistically significant increase in lymphocytes, eosinophils, and large unstained cells (LUC), was a result of statistically significant increase in leukocytes only for LNT dosed males at 4,000 mg/kg bw/day. This significant increase was explained to be due to atypically low leukocyte values (*i.e.*, outside the HCD lower limit for this parameter) detected in 50% of vehicle control males. Contrary to males, LNT dosed females at 4,000 mg/kg bw/day had statistically significant decreased levels of neutrophils, which were also considered as not test item-related. Platelets were statistically significantly increased for only LNT high dosed males compared with vehicle controls, but this finding was not associated with a dose-response relationship. No other differences were detected in the other measured clotting parameters (partial thromboplastin time and activated partial thromboplastin time) for LNT-treated groups compared with controls. No statistically significant differences were observed at the end of the treatment-free period.

When compared with vehicle controls, there was a statistically significant increase in urine volume for LNT high dosed females and statistically significantly reduced specific gravity for all LNT-treated females at the end of the treatment period. The specific urine gravity was statistically significantly lower in LNT males at 2,500 mg/kg bw/day and 4,000 mg/kg bw/day, although the urine volumes were not affected in these male groups. The authors concluded that these findings are irrelevant and unrelated to LNT administration. In addition, no test item-related kidney effects were noted, and all reported individual values were within the HCD ranges, thus, reflected normal biological variations. There were no toxicological relevant differences in urinalysis values between controls and LNT-treated groups after the recovery period.

It was concluded that LNT treatment did not elicit any signs of adverse toxicity. The NOAEL in this study was established at 4,000 mg/kg bw/day.

6.4 Human Studies

No human studies conducted with LNT were identified in GRN 833 and 923 (U.S. FDA, 2019, 2021). However, many studies have been published that investigated the effects of supplementing infant formula with HiMOs, including LNnT, the constitutional isomer of LNT. The weight of the available evidence (published clinical data) evaluating the safety and tolerance of HiMOs in infants supports the conclusion that LNT is GRAS for use in infant formula at use levels of up to 0.8 g/L. The summaries of these studies are incorporated by reference to previous GRAS conclusions, *i.e.*, 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, 2020d). The results confirm that LNnT is largely well-tolerated in adults and infants, and hence can be considered safe for consumption. No additional studies were identified in the literature as being published subsequent to the most recent LNnT sodium salt GRAS determination (GRN 923).

Similarly, no new studies of LNT administration in adults have been identified in the scientific literature since the most recent LNT GRAS Notice.

6.5 Allergenicity

To check if the extra introduced recombinant proteins in production host INB-LNT_01 could be secreted, bio-informatic analysis on the protein sequences was performed to check for potential signal peptide sequences targeting for protein excretion into the extracellular space (SignalP 5.0; Armenteros *et al*., 2019). Since no SignalP sequences were revealed, none of the recombinant proteins could be identified as a secreted protein. In addition, the complete cell debris is separated from the final LNT product during the different manufacturing steps.

The potential allergenic activity of the extra introduced recombinant proteins in *E. coli K-12 MG1655* to obtain production host INB-LNT_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, at the moment of analysis, 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-LNT_01.

Since lactose is used as substrate in the LNT 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

The safe use of LNT has been approved as a novel food in the European Union (EU) in addition to the previously discussed GRAS conclusions of LNT preparations and other HiMO products. The safety of Glycom’s LNT preparation was reviewed by EFSA in 2019, which prompted the issuance of a Scientific Opinion that concluded LNT was safe for use in a number of food categories, such as food supplements or foods for infants and young children (EFSA, 2019). The Opinion states, “*The intake of LNT from the NF at the proposed use levels is unlikely to exceed the intake level of naturally occurring LNT in breastfed infants on a body weight basis*”, and further, “*The Panel concludes that the NF is safe under the proposed conditions of use for the proposed target populations”.*

On the basis that the LNT ingredient manufactured by Inbiose shares a high degree of similarity to the LNT ingredients from other manufacturers that have been previously concluded to be safe and is intended for the same food uses at the same use levels, previous conclusions on safety can be extended to Inbiose's LNT. Therefore, convening of a GRAS Panel is not considered necessary to support a GRAS conclusion on the basis that this HMO ingredient has been evaluated by multiple GRAS Panels and authoritative bodies, including the U.S. FDA and EFSA.

6.7 Conclusion

Based on the above data and information presented herein, Inbiose has concluded that LNT 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.

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

Part 7. § 170.255 List of Supporting Data and Information

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Last Amended	Section Title
101—Food labeling	101.12	4-1-20	Reference amounts customarily consumed per eating occasion
170—Food additives	170.3	4-1-19	Definitions
	170.30	4-1-19	Eligibility for classification as generally recognized as safe (GRAS)

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February 15, 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 001068

Dear Dr. Anderson,

In reference to your letter dated January 17, 2023, regarding Inbiose's GRAS Notice GRN 001068 for the intended uses of lacto-*N*-tetraose (LNT), I am pleased to provide you with our responses to the Agency's questions in the following document.

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

Kind regards,

Joeri Beauprez
Chief Scientific Officer

Question 1. The intended uses of LNT 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 LNT 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 LNT, 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. The specifications for heavy metals are well above the results of the batch analyses. Please consider reducing these specifications to reflect the results of the batch analyses and to ensure that dietary exposure to heavy metals is as low as possible.

Inbiose highlights that the heavy metal specifications proposed for this GRAS notice are in line with the most recent specifications of other HMOs that have been notified to the U.S. FDA without objection from the agency (e.g. GRN 1016, GRN 1015, GRN 1014, GRN 951, GRN 929, GRN 925, GRN 923, GRN 922). The specifications reported in these GRN notices are specified in Table 1.

Other applications (e.g. GRN 1059) do not provide specification limits for arsenic, cadmium and mercury, they only provide a specification for lead of < 0.1 mg/kg, which is 5 times higher the Inbiose's proposed specification for lead (≤0.02 mg/kg) (Table 1).

Table 1. Specifications reported in previous GRN notices#

GRN no.	Date of filing	Date of closure	**FDA's** Letter	Notifier	Specifications (mg/kg)				Reference
					As	Cd	Pb	Hg	
1068	Aug 16, 2022	On-going	N/A	INBIOSE	≤0.2	≤0.1	≤0.02	≤0.5	Current GRN notice
1059	Jun 8, 2022	Dec 2, 2022	"No questions"	Glycom	NS	NS	≤0.1	NS	Specs: Table 2.3.1-4, p. 10
1016	Sep 24, 2021	Jul 15, 2022	"No questions"	Hansen	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 2, p. 8
1015	Sep 24, 2021	Jul 15, 2022	"No questions"	Hansen	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 2, pp. 8-9
1014	Oct 18, 2021	Jul 15, 2022	"No questions"	Hansen	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 2, pp. 10-11
951	Oct 02, 2021	Aug 12, 2021	"No questions"	Danisco	≤0.2	≤0.05	≤0.05	≤0.1	Specs: Table 3, p. 17
929	Jun 19, 2020	Feb 26, 2021	"No questions"	Jennewein	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 4, p. 7
925	May 15, 2020	Feb 08, 2021	"No questions"	Jennewein	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 3, p. 11
923	May 14, 2020	Feb 02, 2021	"No questions"	Jennewein	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 3, p. 11
922	June 3, 2020.	Apr 23, 2021	"No questions"	Jennewein	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 3, p. 7
921	May 14, 2020	Oct 30, 2020	"No questions"	Jennewein	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 3, p. 12
919	May 12, 2020	Oct 30, 2020	"No questions"	Jennewein	≤0.2	≤0.1	≤0.02	≤0.5	Specs: Table 3, p. 14-15

HMOs indicated in this table, likewise Inbiose's LNT product, were produced from fermentation processes using *Escherichia coli* strains, and using similar production techniques.
N/A: Not Available
NS: Not Specified

Nonetheless, despite the specifications (or their absence) in other GRAS notices, Inbiose would like to reduce the proposed specifications of Arsenic (from initially proposed ≤0.2 mg/kg to ≤0.1 mg/kg); Cadmium (from initially proposed ≤0.1 mg/kg to ≤0.05 mg/kg) and Mercury (from initially proposed ≤0.5 mg/kg to ≤0.25 mg/kg). As for Lead, Inbiose would like to keep the initial proposed specification of ≤0.02 mg/kg.

Question 3. Inbiose identifies ultra-high performance liquid chromatography coupled with a refractive index detector (UPLC-RI) as the method of analysis for LNT and other carbohydrates present in Inbiose's LNT ingredient.
a. For the identity specification, please clarify how "conforms" is assessed (e.g., by refractive index, retention time, comparison with known standards).
b. Please confirm that the method includes resolution and identification of "other carbohydrates" present in the LNT ingredient.
c. Please state the identity of the "other carbohydrates" present in the LNT ingredient.

Inbiose confirms that UPLC-RI was the method used for the analysis of carbohydrates in the LNT product.

a. For the identity specifications, conformity was assessed based on the comparison of retention times with know-standards.
b. Inbiose confirms that the method has the required resolution to ensure separation and identification of the "other carbohydrates" from LNT, lactose, LNT-fructose, Lacto-N-triose and Para-Lacto-N-Hexaose (pLNH).
c. ''Other carbohydrates'' that may be also be present as traces in Inbiose's LNT product have been identified as glucose, N-acetylglucosamine (GlucNac), LNT-fructose and pLNH.

Question 4. Inbiose provides a specification for aflatoxin M1 of not more than (NMT) 0.25 µg/kg. We would not expect aflatoxin to be present in LNT produced by controlled fermentation using an *E. coli* K-12 based production host. Please confirm that, based on the production organism and use of food-grade starting materials in the fermentation medium, aflatoxin M1 is not expected to be present in the LNT ingredient.

Inbiose confirms that, based on the controlled fermentation of an *E. coli* K-12 production host and the use of food-grade starting materials in the fermentation medium, aflatoxin M1 is not expected to be present in the final Inbiose´s LNT product.

Question 5. On pages 12-13 of the notice, Inbiose includes a process flow diagram and a brief description of the method of the post-fermentation purification process. For clarity, please expand the narrative to include other minor impurities (e.g., metals, residual DNA, other carbohydrates) that may be removed by the nanofiltration and activated charcoal/resin stages of purification or other steps following filtration of large molecules.

As described in the section 2.2.3 of the GRAS Notice, the post-fermentation product purification process comprises of the steps described in the process flow diagram (Figure 2.2.3-2). Briefly, the biomass is removed by separating the solid material from the liquid by means of micro- and, subsequently, ultrafiltration. This ensures the removal of the production microorganism, protein,

DNA and endotoxins. Heavy metals are removed during the ion chromatography. Following the ion chromatography, the retentate is concentrated by reverse osmosis. Remaining salts and small carbohydrates present in the liquid are removed by nanofiltration (purification and concentration step). Next, the liquid containing LNT with very little salt is again concentrated by evaporation. The color is removed by activated charcoal treatment and subsequently ion chromatography to ensure traces of heavy metals are removed. The microbial quality is guaranteed by sterile filtration prior to drying. The concentrated syrup is filtered after which it is transferred to a bag-in-box system. Bag-in-box and dryer are directly connected to prevent potential (microbial) contamination. A sample is taken from the syrup to assess if the microbiological, organic and inorganic parameters are within specification.

Question 6. 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 LNT 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 7. 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 LNT 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-LNT_01 production strain was originally established, a batch of cryovials was collected from the strain for LNT production. The following quality checks are performed on randomly selected cryovial 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, followed by 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 LNT production after 72 hours of growth.

After the cryovial quality is verified, fermentations with INB-LNT_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-LNT_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-LNT_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 LNT 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-LNT_01. No evidence of significant mutation was observed in any of the five samples collected post-fermentation relative to the production strain INB-LNT_01. All mutations identified in post-fermentation samples were comparable to those of the INB-LNT_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 8. 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 LNT ingredient subject to this GRAS Notice is produced using a non-pathogenic and non-toxigenic production organism.

Question 9. In Table 2.3.1-1, Inbiose provides a specification for LNT-fructose. Please provide a brief description of how the LNT-fructose isomer is formed and why it is not expected to pose a safety concern when consumed by infants and young children.

LNT-fructose isomer is formed by the terminal isomerization of glucose to a fructose. This process is known as the Lobry de Bruyn-van Ekenstein transformation. The isomerization of the glucose sugar to fructose is pH- and temperature-dependent, i.e. at high pH and temperature, the isomerization increases (Schuster-Wolff-Bühring et al, 2010; de Segura et al, 2012). To assure the specification levels of LNT-fructose (i.e. $\leq$ 1.0 % (w/w), the pH and temperature are closely monitored during the downstream process (DSP). Nonetheless, the LNT-fructose isomer formation is unavoidable, and this isomer is produced in very low quantities during DSP. In addition, the isomerization of the terminal glucose moiety to fructose can be observed for all lactose-based HMOs. In a randomized, controlled clinical study, the safety and tolerance of infant formula supplemented with 5 HMOs mixture, including LNT (contained $\leq$ 1.0 % (w/w) LNT-fructose isomer, GRN 923), were evaluated by Parschat

et al. (2021). The results showed that the supplement of HMOs supported the normal infant growth and did not trigger any safety issues. Therefore, it can be concluded that the presence of LNT-fructose at the level of ≤ 1.0 % (w/w) is not expected to pose a safety concern when consumed by infants and young children.

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de Segura, A. G., Escuder, D., Montilla, A., Bustos, G., Pallás, C., Fernández, L., Corzo, N., & Rodríguez, J. M. (2012). Heating-induced bacteriological and biochemical modifications in human donor milk after holder pasteurisation. Journal of pediatric gastroenterology and nutrition, 54(2), 197–203. https://doi.org/10.1097/MPG.0b013e318235d50d

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Question 10. On page 18, Inbiose discusses the stability studies from GRN 000833, noting an analysis for lactulose was performed by the notifier. Given that lactulose can have a laxative effect, please confirm that you do not expect lactulose to be present in the Inbiose ingredient. If lactulose is present, please confirm that the levels are consistent with those reported in GRN 000833. If Inbiose expects lactulose to be formed at levels higher than those levels stated in GRN 000833, please discuss why this is not a safety concern.

The presence of lactulose, a galactose and fructose disaccharide obtained from isomerization of lactose may occur during the downstream processing of Lacto-N-tetraose. This process is pH- and temperature-dependent, i.e. at high pH and temperature, the isomerization increases (Schuster-Wolff-Bühring et al, 2010; de Segura et al, 2012). Under controlled pH and temperature production process, the isomeration of lactulose is very low to absent.

Inbiose confirms that lactulose was not detected by UPLC-RI in Inbiose´s LNT product above the LOQ (i.e. 0.01 % dry matter).

References

de Segura, A. G., Escuder, D., Montilla, A., Bustos, G., Pallás, C., Fernández, L., Corzo, N., & Rodríguez, J. M. (2012). Heating-induced bacteriological and biochemical modifications in human donor milk after holder pasteurisation. Journal of pediatric gastroenterology and nutrition, 54(2), 197–203. https://doi.org/10.1097/MPG.0b013e318235d50d

Schuster-Wolff-Bühring, R., Fischer, L., & Hinrichs, J. (2010). Production and physiological action of the disaccharide lactulose. International Dairy Journal, 20(11), 731-741.

Question 11. On page 36, regarding the 90-day subchronic toxicity study, Inbiose states, "This study is ongoing, and a draft report is not available at the time of submitting this notice." Thus, Inbiose's GRAS conclusion was made prior to knowing the results of this study. Please provide a summary of the results from this 90-day study and discuss whether these results support Inbiose's GRAS conclusion for the proposed uses of LNT. We note that since Inbiose acknowledged the existence of this study in the current notice, we will not be able to complete our evaluation until Inbiose provides information regarding the outcome of the study and Inbiose's safety conclusions.

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 LNT and to support premarket approvals in global jurisdictions where such studies may be necessary (Bentz, 2022 [final report, unpublished]). This study was conducted in accordance with the OECD Test Guideline 408 (2018).

In the main study, groups of 20 (10/sex/group) juvenile Sprague-Dawley rats were administered Inbiose´s LNT 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 96. 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) in the recovery phase, during which the rats were kept un-dosed for additional 4-week treatment-free period, to assess whether any effects observed at the end of the dosing phase persist, partially or fully recover. Finally, another 6 animals (3/sex/group) were included for toxicokinetic evaluation in each of the 0, 1,500, 3,000, or 5,000 mg LNT/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. In addition, the adrenal glands from all animals (males and females), and the kidneys from all females euthanized at the end of the treatment period and at the end of the recovery period were reviewed.

There were no LNT- or FOS-related deaths during the study. No LNT- or FOS-related effects were noted on the hematology, coagulation, blood biochemistry or urinary parameters, or on thyroid hormone levels. No toxicologically significant effects on long bone growth (tibia length), functional observation battery parameters (*i.e.*, reflexes, motor activity, learning/memory function), reproductive development and neurotoxicity or ophthalmology were observed in LNT- or FOS-tested groups.

Administration of LNT induced fully reversible decreased adrenal weights at 3,000 and 5,000 mg/kg body weight/day in both males and females, correlating with minimal, also fully reversible, adrenal cortical atrophy at 5,000 mg/kg body weight/day in both sexes. Similar changes in absolute adrenal weights were also observed in rats administered the FOS reference treatment at 5,000 mg/kg body

weight/day. FOS administration was observed to induce non-adverse decreased adrenal weights in both sexes, correlating with non-adverse adrenal cortical atrophy in females only; however, no macroscopic changes related to FOS treatment were observed. This finding in the adrenal glands was not considered to be adverse by the study investigators given its low magnitude in all LNT- and FOS-affected groups.

In the kidneys, a higher incidence and severity of mineralization at the corticomedullary junction was observed in females administered 5,000 mg LNT/kg body weight/day when compared with control animals. This finding was present in one sex only and was observed with similar incidence and severity in recovery female controls. A relationship with LNT administration was considered by the study investigators to be unlikely, as spontaneous mineralization in the kidneys of young and old rats can regularly be observed, and these lesions are often thought to be influenced by diet (Lord and Newberne, 1990, McInnes, 2012). Moreover, female rats tend to be more affected by corticomedullary junction mineralization than male rats, which may be related to the influence of estrogens (Hard *et al*., 1999; Ritskes-Hoitinga *et al*., 1989; Frazier *et al*.,2012). Of note, the incidence of mineralization in the kidneys in the female control animals included in this 90-day study was also higher than what has been observed in historical control data of Charles River Laboratories Evreux. These data indicate that the apparent increased incidence in treated animals is also likely to be incidental and related to a high incidence of kidney mineralization in this particular batch of animals. These changes, which did not influence the outcome of the study, were observed in other female rats obtained from the same breeder and used in concomitant studies conducted at Charles River Laboratories Evreux.

Other organ weight changes were not considered to be related to LNT/FOS treatment as they were small in amplitude, had no gross or microscopic correlates, were not dose-related in magnitude, and/or were not consistent for the 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 LNT 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 GRN.

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_LNT".**

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Question 12. Since each GRAS notice must independently support the safety of an ingredient for its intended use, please provide brief summaries of the clinical studies that Inbiose is incorporating into the notice in Section 6.4. As part of this discussion,
we suggest including the infant population, study treatments including concentrations of the HMO used in the test formula, and the outcome measures of the study as they relate to Inbiose's conditions of use.

The incorporated clinicals studies using LNnT, the constitutional isomer of LNT, in section 6.4 of the GRAS Notice were summarized by applicant below:

Iribarren et al. (2021) studied the effects of supplementation of two blend of HMOs (2'-FL and LNnT, in a 4:1 ratio, respectively) on adults with irritable bowel syndrome (IBS) in a parallel, randomized, double-blind and placebo-controlled study. Adult patients (N=58, males and females) with IBS symptoms of at least moderate severity received daily placebo (5 g of glucose), 5 g 2'-FL/LNnT, or 10 g 2'-FL/LNnT over a 4-week treatment period. The fecal samples were analyzed at baseline and upon test completion. The mucosal colonic biopsies were taken during sigmoidoscopy. The increasement of relative abundance of specific bacterial taxa was observed in the fecal and mucosal samples, specifically fecal and mucosal *Bifidobacterium adolescentis* and mucosal *Bifidobacterium longum*. Moreover, the HMO supplementation modulated the fecal and plasma metabolite profiles. The authors concluded that 2'-FL/LNnT supplementation shaped the abundance of bifidobacterial and modulated the intestinal microenvironment of patients with IBS.

Fonvig et al. (2021) evaluated safety and digestive tolerance of HMOs (4.5g, 2'-FL or mixture of 2'-FL and LNnT in a mass ratio of 4:1) in children with overweight. In this prospective, randomized, double-blinded, placebo-controlled clinical trial, children were assigned to three groups and randomized to receive either a daily dose of 4.5 g of placebo (powdered glucose), 2'-FL alone, or the Mix for 8 weeks. Safety was assessed via the measurements of a range of biochemical markers (i.e., of inflammatory blood markers, gut barrier integrity proteins, metabolic biomarkers) and occurrence of adverse events or gastrointestinal symptoms. There were no significant differences measured in any of the biochemical parameters and the occurrence of adverse events. Neither test article was observed to induce digestive tolerance issues as assessed by the Gastrointestinal Symptoms Rating Scale. Minor differences in bloating were noted between groups at the trial's midway point, which were considered by the authors to be clinically irrelevant. The authors concluded that the products did not induce digestive tolerance issues and any safety concerns.

In a controlled, double-blind, randomized clinical trial, infants with cow's milk protein allergy (CMPA) were allocated to a test group (N=71) and were fed with extensively hydrolyzed formula (EHF) with reduced protein content (2.20 g/100 kcal) supplemented with LNnT and 2'-FL (0.5 g/L and 1.0 g/L, respectively) (Vandenplas et al., 2022). Another group of infants served as reference group (N=71) and received EHF without HMOs. The weight gain per day and other growth parameters (such as weight, length, head circumference) were measured from enrollment to 4 months' follow-up. The incidence of infections (respiratory, gastrointestinal, and other) and medication use (antibiotics,

antipyretics) were analyzed from enrollment to 12 months of age. At the 4-month follow-up, daily weight gain for the test formula was considered non-inferior to the control formula. Also, no significant group differences in anthropometric parameters were noted. The relative risk of lower respiratory tract and gastrointestinal infections were reduced by 30–40% when compared to the reference group. Therefore, HMO-supplemented formula was shown to support normal growth and was able to protect infants with CMPA from respiratory and ear infections in the first year of life.

Hascoët et al. (2022) performed a randomized, double-blind, placebo-controlled trial conducted in pre-term infants (27–33 weeks of the gestation, birth weight <1,700 g) with HMO supplementation. The preterm infants (N=43) received liquid supplement containing LNnT and 2'-FL in a 1:10 ratio (0.034 and 0.374 g/kg body weight/day, respectively) as soon as 24h of trophic feeding was possible. The supplementation was given until discharge from the neonatal unit. In the control group, the preterm babies (N=43) were provided with only glucose (0.140 g/kg/day). The anthropometric z-scores and feeding tolerance, measured by non-inferiority (NI) in days to reach full enteral feeding (FEF), were analyzed. Significantly higher length-for-age z-scores were observed in HMO-fed group at FEF day 14 ($p = 0.037$) and 21 ($p = 0.037$). Head circumference-for-age z-score was significantly higher in HMO vs. placebo groups at discharge [$p = 0.007$]. There were no significant differences in occurrences of illness and infection as well as the AEs between two groups. The authors concluded that HMO supplementation was safe and well-tolerated in pre-term infants.

In an open-label, non-randomized, multicenter trial study, infants with CMPA received amino acid-based formula (AAF) supplemented with LNnT and 2'-FL (0.5 g/L and 1.0 g/L, respectively; N= 29) (Gold et al., 2022). Growth parameters, tolerability, gut microbiome were studied over a 4-month period. The results showed that the mean weight-for-age Z score (WAZ) increased from –0.31 at the baseline to +0.28 at the 4-months' follow-up. The AAF contained 2'-FL and LNnT was well-tolerated and it was safe to the infants with CMPA. Moreover, the HMO-supplemented study formula was able to partially improve the gut microbial dysbiosis in infants with CMPA by significant enrichment of the HMO-utilizing bifidobacteriaceae and decrease in abundance of fecal Proteobacteria.

In addition, the applicant identified newly published clinicals studies conducted with LNT, please refer to the applicant response to question 13 and Annex I. Summaries of Newly Identified Clinical Trials Conducted with LNT.

References

Fonvig, C. E., Amundsen, I. D., Vigsnæs, L. K., Sørensen, N., Frithioff-Bøjsøe, C., Christiansen, M., Hedley, P. L., Holm, L. A., McConnell, B., & Holm, J. C. (2021). Human Milk Oligosaccharides Modulate Fecal Microbiota and Are Safe for Use in Children With Overweight: A Randomized Controlled Trial. Journal of Pediatric Gastroenterology and Nutrition, 73(3), 408–414. https://doi.org/10.1097/MPG.0000000000003205

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Question 13. Please provide an updated literature search for data and information relating to the safety of LNT since the conclusion of Inbiose's literature search in April 2021. As part of this search, please also indicate if any clinical studies have been recently published in which LNT was included in a test formula consumed by an infant population.

To address this question, Inbiose has conducted an updated literature search through February 15 2023 to identify any new publicly available data pertaining to the safety of LNT 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 LNT 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 Annex I for completeness. Briefly, LNT (in combination with another HMOs) was not observed to elicit adverse effects in humans. Results from these recently published studies support that LNT is safe and well tolerated in infants when provided at levels consistent with the proposed uses of Inbiose's LNT described in the GRAS Notification. Inbiose therefore maintains that this LNT 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.

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

the breast-fed group. The authors showed that the mixture of 5 HMOs had positive effect on normal infant growth and was safe and well tolerated for use in healthy term infants.

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

In a randomized, controlled, double-blind trial, Bosheva et al. (2022) investigated the gut maturation effects (microbiota, metabolites, and selected maturation markers) of an infant formula containing a specific blend of five HMOs. Healthy full-term infants were assigned to control group (CG) fed a standard IF without HMOs, test group 1 (TG1) and test group 2 (TG2) fed with the same standard IF containing the five-HMO blend at a concentration of 1.5 g/L and 2.5 g/L, respectively. A non-randomized human milk-fed infants (HMG) served as reference group. Fecal samples collected at baseline, age 3 and 6 months, were analyzed for microbiome (shotgun metagenomics), pH and organic acids, as well as the biomarkers (immunoglobulin A (sIgA), calprotectin and alpha-1-antitrypsin). Higher bifidobacterial and lower toxigenic C. difficile abundance were observed in the TGs vs. CG. Early life intestinal immune response was improved as indicated by the higher fecal sIgA concentration in the TGs vs. CG. The authors concluded that the infant formula contained specific HMO blend was able to support the development of the intestinal immune system, and shaped the gut microbiota directionally toward that of breastfed infants.

References

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

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

Question 14. On page 24, Inbiose cites GRN 000923 and discusses the concentrations of LNT in human milk to support the use level of 0.8 g/L in infant formula. Since the proposed use level of 0.8 g/L of LNT in infant formula has previously been concluded to be GRAS, we note the following for informational purposes only and do not require a response: Additional information on LNT concentrations in human milk have been published in the literature, including systematic reviews, since the closure of GRN 000923; we suggest including this information in future GRAS notices for HMO ingredients.

Inbiose appreciates the suggestion provided by FDA and will take it into consideration in future GRAS notices for HMO ingredients.

Additional corrections:

Inbiose took this opportunity to revise the content of the GRAS Notice 001068.

A. Heavy metal values in Table 2.3.2-1

Inbiose would like to inform that the Heavy metals values of Table 2.3.2-1 have been wrongly reported by the external lab performing the Heavy Metal analysis on two batches: ilex14F03 and ilex14F04. The external lab stated that:

> ''*Previously reported LOQs were incorrect due to a technical problem with Primoris' automatic reporting of the LOQs for heavy metals.*
> *Therefore, this report is regenerated with the correct and current LOQs.*''
>
> Therefore, Inbiose would like to update the section of Heavy metals indicated in Table 2.3.2.1 of this GRN 001068, specifically Cadmium, Lead and Mercury, with the correct values (in blue) shown in Table 2 below.

Table 2. Corrected heavy metal values

Parameter	**Specification #**	**Lot Nos.**				
		ilex14F03	**ilex14F04**	**ilex14F05**	**ilex14F10**	**ilex14F11**
Heavy Metals						
Arsenic (mg/kg)	≤0. 1	<0. 01	<0. 01	<0. 01	<0. 01	<0. 01
Cadmium (mg/kg)	≤0. 05	<0. 005	<0. 005	<0. 005	<0. 005	<0. 005
Lead (mg/kg)	≤0. 02	<0. 01	<0. 01	<0. 01	<0. 01	<0. 01
Mercury (mg/kg)	≤0. 25	<0. 01	<0. 01	<0. 01	<0. 01	<0. 01

Proposed specification limits of heavy metals, see the response to Question 3

B. Ash content specification

For consistency with specifications reported for other LNT products (GRN 000833 and GRN 000923, see Table 2.3.1-1 of this GRN 0001068, page 14), Inbiose would like to change the ash specification to no more than (NMT) 0.5% w/w, see Table 3 below with the updated specification (in blue).

Table 3. Updated ash content product specifications for Inbiose's LNT in comparison to those of the LNT Ingredients in GRN 833 and 923

Parameter	Specification for Inbiose's LNT	Method of Analysis Employed by Inbiose	Specifications Reported for Other LNT Products	
			Glycom's LNT (GRN 833) (U.S. FDA, 2019a)	Jennewein's LNT (GRN 923) (U.S. FDA, 2021)
Chemical Analysis				
Ash content	NMT 0.5% w/w	NEN 6810 (500-550°C)	NMT 0.5 w/w %	NMT 1% (w/w)

C. Residual endotoxins specification

Minor corrections are necessary with respect to the Residual endotoxins specification presented in the table Table 2.3.1-1 of this GRN 001068, page 15. The specifications for residual endotoxin levels were erroneously expressed as EU/g. The corrected unit is EU/mg, as indicated in Table 4 below with the corrected units (in blue).

Table 4. Corrected Residual endotoxins specifications for Inbiose's LNT in comparison to those of the LNT Ingredients in GRN 833 and 923

Parameter	Specification for Inbiose's LNT	Method of Analysis Employed by Inbiose	Specifications Reported for Other LNT Products	
			Glycom's LNT (GRN 833) (U.S. FDA, 2019a)	Jennewein's LNT (GRN 923) (U.S. FDA, 2021)
Chemical Analysis				
Residual endotoxins	NMT 10 EU/mg	Ph. Eur. 2.6.14	NMT 10 EU/mg	NMT 10 EU/mg

Annex I. Summaries of Newly Identified Clinical Trials Conducted with LNT

Type of Study	Population	Length of Study	Dose	Result	Reference
Randomized, controlled, parallel-group clinical study	Term, healthy infants ≤14 days of age	112 ± 3 days	Infant formula mixed with 5HMO-Mix (2.99 g/L 2'-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3'-SL, and 0.28 g/L 6'-SL) (N = 86) Control formula without HMOs (N = 91) Placebo: Breast milk (N = 88)	The results demonstrated that 5 HMO-Mix at 5.75 g/L in infant formula was safe and well tolerated by healthy term infants during the first months of life.	Parschat et al., 2021
Randomized, double-blind, controlled parallel feeding growth trial	Healthy term infants (gestational age 37–42 weeks) between 0 and 14 days of age with a birth weight ≥ 2490 g.	Time of enrolment at ≤14 Days (D) of age until D 119 or up to D 183	Control milk-based formula (CF; n = 129); experimental formula (EF; N = 130) containing five HMOs (5.75 g/L; 2'-FL (3.0 g/L), 3-FL (0.8 g/L), LNT (1.5 g/L), 3'-SL (0.2 g/L) and 6'-SL (0.3 g/L); reference group: human milk (HM; N = 104)	No significant differences among the three groups for weight gain per day and gains in weight and length (p ≥ 0.05) from D 14 to D 119. Color of stool, its consistency and frequency per day were more similar between EF and HM groups. Serious and non-serious adverse events were not different among groups. The results indicated that EF containing five HMOs was safe and well-tolerated and supported age-appropriate growth.	Lasekan et al., 2022

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

3. SUMMARY

The objective of this study was to evaluate the potential toxic effects of the test item, Lacto-N-Tetraose (LNT), on the development of juvenile rats, following daily oral administration from post-natal Day (PND) 7 to at least PND 96 (at least 90-day treatment period). The study was intended to cover the period of development corresponding to infancy, childhood and adolescence.

On completion of the treatment period, designated animals (control and high-dose level groups) were held for a 4-week treatment-free period in order to evaluate the reversibility of any findings.

In addition, satellite animals were dosed for toxicokinetic assessment.

Methods

Four groups of 10 male and 10 female juvenile Sprague-Dawley rats received the test item, Lacto-N-Tetraose (LNT) (batch No. ilex14F03), daily by the oral route (gavage), at the dose level of 0, 1500, 3000 or 5000 mg/kg/day, from PND 7 to at least PND 96. The test item was administered in sterile water for injection under a constant dosage volume of 10 mL/kg/day. Another group of 10 males and 10 females received the reference item, Fructooligosaccharides, (FOS, Actilight 950P) (batch No. 0175092941), at 5000 mg/kg/day, under the same experimental conditions.

In addition, five animals/sex in the control- and high-dose test item-treated groups were also dosed under the same conditions and then retained untreated for 4 weeks in order to assess recovery of any test item-related effects.

Satellite animals were included in control and test item-treated groups (three males and three females per group) for the determination of plasma and urine test item levels at the end of the treatment period.

The actual test or reference item concentrations in the dose formulations were determined on four occasions during the study.

The animals were checked at least twice daily for mortality and at least once daily for clinical signs. Body weight and food consumption were recorded twice weekly from weaning until the end of the study.

The length of the tibia was measured from PND 7, every two days during lactation and then weekly after weaning until the end of the treatment period.

All animals were assessed for pre-weaning development, including eye opening, tooth eruption, auditory canal opening, air righting test and cliff avoidance.

Functional observation battery, reflexes, motor activity, and learning and memory (5-T Biel water maze) were evaluated at the end of the treatment period for ten animals per sex and group.

Reproductive development [cleavage of the balanopreputial groove (preputial separation) or vaginal opening] was observed in ten males per group every day from PND 40 until positive, and in 10 females per group every day from PND 28 until positive.

An ophthalmological examination was performed on all animals at the beginning of the post-weaning period and on ten animals per sex and group at the end of the treatment period.

Estrous cycle stage was determined for 5 consecutive days at the end of the treatment period in ten females per group.

Blood and urine for hematology, coagulation, blood biochemistry and urinary investigations were collected in the animals euthanized at the end of the treatment period. Hematology and blood biochemistry investigations were also performed at the end of the treatment-free period. Determination of thyroid hormone levels was performed at the end of the treatment period.

Seminology investigations (spermatozoa count, motility and/or morphology) were performed on all principal males before scheduled euthanasia at the end of the treatment period.

Animals were euthanized on completion of the treatment or treatment-free period and a complete macroscopic *post-mortem* examination was performed. Selected organs were weighed and preserved. A microscopic examination was performed on selected tissues from the control, reference and high-dose animals euthanized at the end of the treatment and treatment-free periods, on kidneys (females) and adrenal glands (both sexes) from the low- and mid-dose animals, and on all macroscopic lesions.

Results

Actual concentrations of the test item and reference item in the dose formulations analyzed during the treatment period remained within an acceptable range of variations when compared with the nominal concentrations.

All test item-treated rats were exposed to LNT, with quantifiable plasma concentrations measured up to 4, 6 or 24 hours after administration. The first peak of plasma LNT concentrations was observed 1 to 2 h after administration There was no obvious relationship with the dose-level. At 1500 and 3000 mg/kg/day, no marked differences were observed between males and females, while at 5000 mg/kg/day, males showed higher systemic exposure to LNT than females. In males, an approximately dose-proportional increase in exposure was observed between 1500 and 3000 mg/kg/day, and a trend towards a more than dose proportional increase was observed between 3000 and 5000 mg/kg/day. In females, an approximately dose-proportional increase in exposure was observed over the range of administered doses. Based on the inter animal variability this result should be interpreted with caution.

LNT dose excretion in urine (0-24h interval) was negligible (<0.15% of the dose). Similar levels were observed in males and females at 1500 and 3000 mg/kg/day, while an approximately 2-fold higher excreted amount of LNT was observed in males at 5000 mg/kg/day.

No test or reference item-related deaths occurred during the study.

Occasions of yellowish liquid feces were observed at the beginning of the treatment period (over Study Days 3 to 15) in most males and females treated with the test item at 5000 mg/kg/day or the reference item. This was associated, in reference item-treated animals only, with anal erythema in most animals and anal swelling in 2/10 males. All these findings were considered to be non-adverse.

No or no adverse test or reference item effects were noted on mean body weight, mean body weight change or mean food consumption.

No toxicologically significant test or reference item effects were noted on long bone growth (tibia length), developmental landmarks, neurologic development (functional observation battery parameters, motor activity, learning/memory functions), reproductive development or ophthalmology.

Distribution of estrous stages and seminology parameters were not impacted by the test or reference item treatment.

No test or reference item-related effects were noted on the hematology, coagulation, blood biochemistry or urinary parameters, or on thyroid hormone levels.

Administration of the test item induced fully reversible decreased adrenal weights from 3000 mg/kg/day onwards, correlating with minimal, also fully reversible, adrenal cortical atrophy at 5000 mg/kg/day in both sexes. In addition, when compared with controls, an equivocal increase in the incidence and severity of mineralization was observed in the kidneys at the cortico-medullary junction in females at 5000 mg/kg/day. Mineralization was not increased at the end of the treatment-free period. There were no organ weight or macroscopic changes related to the test item at 1500 mg/kg/day. None of these changes were considered to be adverse.

Administration of the reference item at 5000 mg/kg/day induced non-adverse decreased adrenal weights in both sexes, correlating with non-adverse adrenal cortical atrophy in females only. No macroscopic changes related to the reference item were observed.

Conclusion

The test item, Lacto-N-Tetraose (LNT), was administered once daily from PND 7 to at least PND 96, by oral gavage, to juvenile Sprague Dawley rats at 1500, 3000 or 5000 mg/kg/day. The reference item (for comparison with the highest Lacto-N-Tetraose group), FOS (Actilight 950P), was administered at 5000 mg/kg/day. The study was intended to cover the period of development corresponding to infancy, childhood and adolescence. On completion of the treatment period, designated animals (control and high-dose test item groups) were held for a 4-week treatment-free period in order to evaluate the reversibility of any findings. The test item was clinically well tolerated.

The No Observed Adverse Effect Level (NOAEL) in this study was established at 5000 mg/kg/day for juvenile males and females, based on the absence of adverse effects at this dose level. This dose-level corresponded to an AUC_{0-t} of 44700 ng·h/mL in males and 13300 ng·h/mL in females and a C_{max} of 9760 ng/mL in males and 3790 ng/mL in females at the end of the treatment period.

From: Joeri Beauprez
To: Anderson, Ellen
Cc: Kamila Solak - Inbiose
Subject: [EXTERNAL] Re: GRN 001068 administrative clarification
Date: Tuesday, May 2, 2023 10:56:55 AM
Attachments: image001.png
Outlook-lj043r1h.png

Dear Dr Anderson,

Thank you for your e-mail. Hereby Inbiose confirms, that Inbiose does not intend for LNT to be used in '*other baby foods for infants and young children*' under the jurisdiction of the United States Department of Agriculture.

Kind regards
Joeri

Joeri Beauprez, PhD

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From: Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>
Sent: 01 May 2023 22:00
To: Joeri Beauprez <Joeri.Beauprez@inbiose.com>
Subject: GRN 001068 administrative clarification

Hello Dr. Beauprez,

We are finishing up our response letter to GRN 001068 and have one administrative detail to clarify with you. Could you please confirm that Inbiose does not intend for LNT to be used in foods under the jurisdiction of the United States Department of Agriculture? We note that this clarification is directed at the intended use of LNT in baby foods for infants and young children.

Sincerely,

Ellen
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Regulatory Review Scientist

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