March 19, 2020

Rachel Morissette, Ph.D.
Regulatory Review Scientist
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
CPK-2 Building, Room 2092
5001 Campus Drive, HFS-225
College Park, MD 20740

Dear Dr. Morissette:

It is our opinion that the enclosed GRAS Determination for the Use of Lacto-N-tetraose (LNT) in Non-Exempt Term Infant Formula constitutes a new notification. Although LNT is the subject of GRAS Notice 833, the subject of the enclosed GRAS Notice is produced using a novel production process and the intended use levels are greater than those that have been determined GRAS.

We thank you for taking the time to review this GRAS determination. Should you have additional questions, please let us know.

Sincerely,

Dietrich B. Conze, Ph.D.
Managing Partner

Enclosure:

CD containing Form 3667, cover letter, GRAS Determination for the Use of Lacto-N-tetraose (LNT) in Non-Exempt Term Infant Formula, and all references

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

GENERALLY RECOGNIZED AS SAFE *(GRAS)* NOTICE (Subpart E of Part 170)

Form Approved: OMB No. 0910-0342; Expiration Date: 09/30/2019
(See last page for OMB Statement)

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000923	Mar 23, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway *(see Instructions)*; OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety *(HFS-200)*, Center for Food Safety and Applied Nutrition, Food and Drug Administration,5001 Campus Drive, College Park, MD 20740-3835.

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

☒ New ☐ Amendment to GRN No. _____ ☐ Supplement to GRN No. _____

2. ☒ All electronic files included in this submission have been checked and found to be virus free. *(Check box to verify)*

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd):* 10/21/2019

4 For Amendments or Supplements: Is your amendment or supplement submitted in response to a communication from FDA? *(Check one)* ☐ Yes ☐ No If yes, enter the date of communication *(yyyy/mm/dd):* _____

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Julia Parkot	Head of Quality Unit and Regulatory Affairs

Organization *(if applicable)*: Jennewein Biotechnologie GmbH

Mailing Address *(number and street)*: Maarweg 32

City	State or Province	Zip Code/Postal Code	Country
Rheinbreitbach	Rheinbreitbach	D-53619	Germany

Telephone Number	Fax Number	E-Mail Address
+49 - (0)2224-98810-251		julia.parkot@jennewein-biotech.de

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Dietrich B. Conze, PhD	Managing Partner

Organization *(if applicable)*: Spherix Consulting Group, Inc.

Mailing Address *(number and street)*: 11821 Parklawn Drive, Suite 310

City	State or Province	Zip Code/Postal Code	Country
Rockville	Maryland	20852	United States of America

Telephone Number	Fax Number	E-Mail Address
240-367-6089		dconze@spherixgroup.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

1. Name of notified substance, using an appropriately descriptive term

Lacto-N-tetraose (LNT)

2. Submission Format: *(Check appropriate box(es))*

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

4. Does this submission incorporate any information in CFSAN's files? *(Check one)*

☒ Yes *(Proceed to Item 5)* ☐ No *(Proceed to Item 6)*

5. The submission incorporates information from a previous submission to FDA as indicated below *(Check all that apply)*

☒ a) GRAS Notice No. GRN 571

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRN 833

6. Statutory basis for conclusions of GRAS status *(Check one)*

☒ Scientific procedures *(21 CFR 170.30(a) and (b))* ☐ Experience based on common use in food *(21 CFR 170.30(a) and (c))*

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information *(Check all that apply)*

☐ Yes, information is designated at the place where it occurs in the submission

☐ No

9. Have you attached a redacted copy of some or all of the submission? *(Check one)*

☐ Yes, a redacted copy of the complete submission

☐ Yes, a redacted copy of part(s) of the submission

☐ No

SECTION D – INTENDED USE

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Jennewein Biotech intends to use LNT as an ingredient in cow's milk-based, non-exempt term infant formula.

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☒ No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

☐ Yes ☐ No , you ask us to exclude trade secrets from the information FDA will send to FSIS.

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)

☒ PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).

☒ PART 3 of a GRAS notice: Dietary exposure (170.235).

☒ PART 4 of a GRAS notice: Self-limiting levels of use (170.240).

☒ PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).

☒ PART 6 of a GRAS notice: Narrative (170.250).

☒ PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

Other Information

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

☐ Yes ☒ No

Did you include this other information in the list of attachments?

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Jennewein Biotechnologie GmbH
(name of notifier)

has concluded that the intended use*(s)* of Lacto-N-tetraose (LNT)
(name of notified substance)

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. Jennewein Biotechnologie GmbH agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

Maarweg 32, D-53619 Rheinbreitbach, Germany
(address of notifier or other location)

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance.The notifying party certifies that the information provided herein is accurate and complete to the best or his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official, Agent, or Attorney	**Printed Name and Title**	**Date (mm/dd/yyyy)**
Dietrich B. Conze, PhD Digitally signed by Dietrich B. Conze, PhD Date: 2020.03.20 12:45:29 -04'00'	Dietrich B. Conze, PhD, Managing Partner	03/19/2020

SECTION G – LIST OF ATTACHMENTS

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Jennewein LNT GRAS Final to FDA.pdf	Submission
	References	Submission

GRAS Determination for the Use of Lacto-*N*-tetraose (LNT) in Non-Exempt Term Infant Formula

Prepared for:

Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

Prepared by:

Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310
Rockville, MD 20852

March 19, 2020

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2’-FL: 2'-Fucosyllactose

3-FL: 3-Fucosyllactose

3’-SL: 3’-Sialyllactose

6’-SL: 6’-Sialyllactose

Alb: Albumin

ALT: Alanine aminotransferase

araA: Arabinose isomerase

BMI: Body mass index

BW: Body weight

CBPI: Cytokinesis-block proliferation index

CFR: United States Code of Federal Regulations

CFU: Colony forming units

CHO: Chinese hamster ovary cells

CI: Confidence interval

COSY: Correlation spectroscopy

DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen

DW: Dry weight

EDI: Estimated Daily Intake

EFSA: European Food Safety Authority

EU: Endotoxin unit

F6PPK: Fructose-6-phosphate phosphoketolase

FCC: Food Chemicals Codex

FDA: United States Food and Drug Administration

FFDCA: Federal Food, Drug, and Cosmetic Act

FOIA: Freedom of information Act

FOS: Fructooligosaccharides

Fru-1,6-BP: Fructose-1,6-bisphosphate

Fru-6-P: Fructose-6-phosphate

FSSC: Food Safety System Certification

FUT: Fucosyltransferase

GI: Gastrointestinal

Glc-1-P: Glucose-1-phosphate

Glc-6-P: Glucose-6-phosphate

Gln-1-P: Glucosamine-1-phosphate

Gln-6-P: Glucosamine-6-phosphate

Glob: Gobulin

GluNAc-6-P: *N*-acetylglucosamine-6-phosphate

GMO: Genetically modified organism

GMP: Good manufacturing practices

GOS: Galactooligosaccharides

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

HCD: Historical control data

HDL-C: High-density lipoprotein cholesterol

HMBC: $^{1}H^{13}C$-heteronuclear multiple bond correlation

HMO: Human milk oligosaccharides

HPAEC-PAD: High performance anion exchange chromatography coupled with pulsed amperometric detection

HSQC: $^{1}H^{13}C$-Heteronuclear single quantum correlation

ICP-MS: Inductively coupled plasma mass spectrometry

IFNγ: Interferon gamma

LC-MS: Liquid chromatography coupled with mass spectrometry

LDL-C: Low-density lipoprotein cholesterol

LDPE: Low-density polyethylene

LNDFHI: lacto-N-difucohexaose I

LNnT: Lacto-*N*-*neo*tetraose

LNT: Lacto-N-tetraose

LOD: Limit of detection

LOQ: Limit of quantitation

MCH: Mean corpuscular hemoglobin

MCV: Mean corpuscular volume

ND: Not detected

NHANES: National Health and Nutrition Examination Surveys

NIH: National Institutes of Health

NMR: Nuclear magnetic resonance

NOAEL: No Observed Adverse Effect Level

OECD: Organization for Economic Cooperation and Development

PCR: Polymerase chain reaction

Ph Eur: European Pharmacopoeia

pLNnH: Para-lacto-N-neohexaose

qPCR: Quantitative polymerase chain reaction

RI: Replicative index

TP: Total protein

UDP-Gal: UDP-galactose

UDP-Glc: UDP-glucose

UDP-GlcNAc: UDP-N-acetylglucosamine

I. SIGNED STATEMENT OF THE CONCLUSION OF GENERALLY RECOGNIZED AS SAFE (GRAS) AND CERTIFICATION OF CONFORMITY TO 21 CFR §170.205-170.260

A. SUBMISSION OF GRAS NOTICE

Jennewein Biotech is hereby submitting a GRAS notice in accordance with subpart E of part 170.

B. NAME AND ADDRESS OF THE SPONSOR

Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

C. COMMON OR USUAL NAME

Lacto-*N*-tetraose (LNT)

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

This notification does not contain any trade secret or confidential information.

E. INTENDED USE

Jennewein Biotech intends to use LNT as an ingredient in cow's milk-based, non-exempt term infant formula.

F. BASIS FOR GRAS DETERMINATION

Lacto-*N*-tetraose for the intended use and use level specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). The safety of the intake of LNT has been determined to be GRAS by demonstrating that the safety of the intended level of intake is generally recognized as safe by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food and is based on generally available and accepted information.

The use of LNT as an ingredient for the intended use in infant formula has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. The subject of this GRAS Notice is a spray-dried, powdered food ingredient that contains not less than 75% LNT dry weight.

    a. LNT is a neutral, non-fucosylated oligosaccharide in human milk.

    b. The LNT that is the subject of this GRAS Notice is structurally identical to the LNT present in human breast milk.

    c. The subject of this GRAS Notice is manufactured by Jennewein in Food Safety System Certification (FSSC) 22000-, ISO 9001:2015-, GMP-, and/or International Featured Standards Food 6.1-compliant facilities. Jennewein is a Food Facility registered with FDA.

    d. The subject of this GRAS Notice is manufactured using a genetically engineered strain of *Escherichia coli* BL21(DE3). Because this organism does not possess the components required for *E. coli* pathogenicity, *E. coli* BL21(DE3) and strains derived from DE3 are non-pathogenic.

    e. All raw materials, processing aids, and food contact substances are GRAS and/or conform to the specifications stated in 21 CFR and/or the Food Chemicals Codex (FCC).

    f. Fermentation by-products include lactose, the intermediate lactose-*N*-triose 2 (LNT II), and para-lacto-*N*-hexaose (pLNH), which are known human milk oligosaccharides; their presence in the finished ingredient is not of toxicological concern.

    g. Product specifications are in place to control the levels of residual impurities and carbohydrate by-products, as well as heavy metals, microbes, and production organism-derived DNA and possible endotoxin, ensuring a consistent, safe, food-grade finished ingredient.

    h. The available stability studies indicate a shelf-life of two years when stored from the date of production under ambient conditions.

2. Human milk oligosaccharides, including LNT, are resistant to the digestive enzymes in the gastrointestinal tract, poorly absorbed, and pass through the gastrointestinal tract where they are either fermented by the microbiota or excreted unchanged.

3. Published studies show that the amount of LNT in breast milk ranges from 0.003 to 6.7 g/L, with means and medians ranging from 0.1 to 3.9 and 0.2 to 2.1 g/L, respectively.

4. Genotoxicology and subchronic toxicology studies published by Phipps et al. (2018) show that LNT is not genotoxic and has a no observed adverse effect level (NOAEL) of 4 g/kg bw/day, which was the highest dose tested.

5. The addition of 1.82 g/L LNT in infant formula will result in mean and 90th percentile Estimated Daily Intakes (EDIs) of 2.52 and 4.95 g/day (0.4 and 0.7 g/kg bw/day), respectively, in infants 0 to 6 months-of-age, which represents the maximum level of intake because infants at this age consume infant formula as a sole source of nutrition.

6. The safety of exposure to Jennewein's LNT at its intended use level is supported by:

    a. Published studies that quantitate the levels of LNT in human milk;

    b. Analytical data demonstrating that the LNT produced by Jennewein is structurally identical to LNT from human milk;

    c. Data demonstrating the qualitative and quantitative similarities between the subject of this GRAS Notice and the LNT ingredient tested by Phipps et al. (2018), which is the subject of GRN 833;

    d. Corroborative genotoxicology and 90-day subchronic dietary toxicology studies conducted with a mixture of human milk oligosaccharides published by Parschat et al. (2020), which contained 26% (dry weight) of Jennewein's LNT).

    e. A corroborative unpublished tolerance study in neonatal piglets conducted with a mixture of HMOs containing up to 1.6 g/L of Jennewein's LNT ingredient that showed an HMO mixture containing LNT was well-tolerated and supported normal growth in neonatal piglets.

Therefore, LNT is safe and GRAS at the proposed level of addition to the intended infant formula. Lacto-*N*-tetraose is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

G. PREMARKET APPROVAL

The notified substance is not subject to the premarket approval requirements of the FD&C Act based on our conclusion that the substance is GRAS under the conditions of intended use.

H. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Dietrich Conze, PhD, Managing Partner, Spherix Consulting Group Inc., at 11821 Parklawn Drive, Suite 310, Rockville, MD 20852; Telephone: 240-367-6089; Email: dconze@spherixgroup.com; or be sent to FDA upon request.

I. FREEDOM OF INFORMATION ACT (FOIA)

Parts 2 through 7 of this notification do not contain data or information that is exempt from disclosure under the FOIA.

J. INFORMATION INCLUDED IN THE GRAS NOTIFICATION

To the best of our knowledge, the information contained in this GRAS notification is complete, representative and balanced. It contains both favorable and unfavorable information, known to Jennewein Biotechnologie GmbH and pertinent to the evaluation of the safety and GRAS status of the use of this substance.

[redacted] 19/3/20

Signature of Authorized Representative of Jennewein Biotechnologie GmbH — Date

II. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE

A. COMMON OR USUAL NAME

Lacto-*N*-tetraose (LNT; CAS No. 14116-68-8)

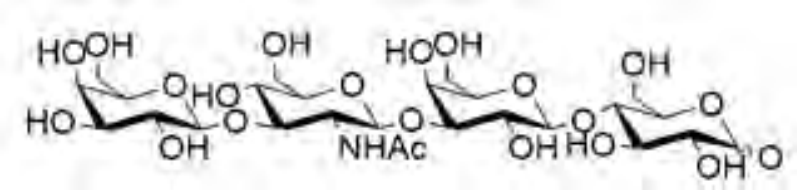
B. CHEMICAL NAME

β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose

C. MOLECULAR FORMULA AND MASS

$C_{26}H_{45}NO_{21}$; 707.632 Da

D. STRUCTURAL FORMULA



E. DESCRIPTION OF LACTO-N-TETRAOSE

Lacto-*N*-tetraose is one of the most abundant oligosaccharides in human milk. It is one of the most abundant core structures in human milk and can be further decorated by the addition of fucosyl or sialyl residues, resulting in over 200 different HMOs identified to date. Lacto-*N*-tetraose is a linear tetrasaccharide consisting of a terminal D-galactose linked through a β-(1→3) bond to *N*-acetyl-D-glucosamine (GlcNAc), linked through a β-(1→3) bond to D-galactose, linked through a β-(1→4) bond to the reducing end D-glucose (see Structural Formula). It is also an isomer of LNnT (lacto-*N*-*neo*tetraose), which contains the same monosaccharide moieties with the linkage between the terminal galactose and GlcNAc being β-(1-4) and considered to be a Type I oligosaccharide due to the presence of the characteristic Galactose (Gal) (β1→3) *N*-acetyl-D-glucosamine [lacto-*N*-biose I (LNB)] unit (Urashima et al., 2012).

The subject of this GRAS Notification is produced by fermentation using a genetically engineered strain of *Escherichia coli* BL21 (DE3). The ingredient is then purified from the culture medium and residual impurities include lactose and carbohydrate by-products. Importantly, the structure of the LNT that is the subject of this GRAS notification is consistent with the structure of LNT as confirmed by liquid chromatography coupled with tandem mass spectrometry (LC-

MS/MS), ^{1}H, ^{13}C, double-quantum filtered $^{1}H^{1}H$-COSY, phase-sensitive $^{1}H^{13}C$-heteronuclear single quantum correlation (HSQC), phase-sensitive $^{1}H^{13}C$-heteronuclear multiple bond correlation (HMBC) NMR spectroscopy. Additionally, another ingredient containing LNT produced by fermentation is GRAS in the United States (GRN 833).

F. PRODUCTION PROCESS

LNT is produced by fermentation using *JBT-LNT*, which is a genetically engineered strain of *E. coli* BL21 (DE3) that secretes the LNT into the fermentation medium. The LNT is then purified from the fermentation medium and spray-dried, producing the powdered LNT.

1. Description of the Production Strain

To generate *JBT-LNT*, a Basic strain was first engineered to allow for the subsequent engineering needed to generate *JBT-LNT*. All genes integrated into the basic and *JBT-LNT* strains are well-characterized. Additionally, all plasmids and/or episomal vectors were removed during the engineering process. Both strains are stored at the production site as glycerol stocks in a master cell bank at -80°C. All strains will be deposited at the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen)-German Collection of Microorganisms and Cell Cultures. The glycerol stocks are used to produce working cell banks, which are then used to the production of the finished ingredient.

a. The Basic Strain

To generate the Basic strain, endogenous genes encoding a β-galactosidase, L-arabinose-isomerase, L-fucose isomerase, L-fuculokinase, *N*-acetylglucosamine 6-phosphate deacetylase, glucosamine 6-phosphate deaminase, lipopolysaccharide biosynthesis protein, and UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase were either inactivated by mutagenesis using mismatched oligonucleotides or deleted by homologous recombination (Table 1). In contrast, genes encoding a UDP-galactose-4-epimerase, galactosyltransferase, galactokinase, galactose mutarotase, and lactose permease were amplified from *E. coli* K12 genomic DNA and integrated by either site-specific homologous recombination or transposition (Table 1) (Datsenko and Wanner, 2000). Arabinose isomerase (*ara*A) was inactivated by mutagenesis using mismatch oligonucleotides to prevent L-arabinose degradation (Ellis et al., 2001) and allow for arabinose-induced expression of λ red recombinase and transposase required for transposition. The antibiotic resistance genes that were integrated during homologous recombination or transposition, and used for selection of the recombinants, were then removed from the genome by plasmid and Cre-mediated recombination (Datsenko and Wanner, 2000). All gene deletions and insertions were verified by PCR using oligonucleotides specific to the coding

sequence and Basic strain genomic DNA. Loss of the plasmids used to express λ red recombinase, transposase and Cre recombinase, all of which contained ampicillin resistance genes and temperature-sensitive origins of replication, was confirmed by ampicillin sensitivity after incubation at 42°C, and failure to amplify plasmid specific DNA.

Table 1. Genetic Manipulations in Basic Strain

Gene Product Name	Origin of the Gene	Manipulation	Effect
β-galactosidase	*E. coli* BL21(DE3)	Deletion	To prevent lactose hydrolysis
Arabinose isomerase	*E. coli* BL21(DE3)	Inactivation	To prevent arabinose degradation
L-fucose isomerase	*E. coli* BL21(DE3)	Deletion	To prevent fucose degradation
L-fuculokinase	*E. coli* BL21(DE3)	Deletion	
N-acetylglucosamine-6-phosphate deacetylase	*E. coli* BL21(DE3)	Deletion	To prevent *N*-acetyl-glucosamine catabolism
Glucosamine-6-phosphate deaminase	*E. coli* BL21(DE3)	Deletion	
Lipopolysaccharide biosynthesis protein	*E. coli* BL21(DE3)	Deletion	To prevent colonic acid synthesis
UDP-glucose:undecaprenyl phosphate glucose-1 phosphate transferase	*E. coli* BL21(DE3)	Deletion	
Lactose permease	*E. coli* K12	Ectopic expression	Facilitate lactose uptake
UDP-galactose-4-epimerase	*E. coli* K12	Ectopic expression	To allow for galactose utilization.
Galactosyltransferase	*E. coli* K12	Ectopic expression	
Galactokinase	*E. coli* K12	Ectopic expression	
Galactomutarotase	*E. coli* K12	Ectopic expression	

b. JBT-LNT

The production strain *JBT-LNT* was generated from the Basic strain to allow for the synthesis of the intermediate lacto-*N*-triose (LNT II) from lactose. Specifically, UDP-galactose and UDP-*N*-acetylglucosamine synthesis pathway genes, a lactose-specific *N*-acetylglucosaminyltransferase, and a β-1,3-galactosyltransferase were integrated by transposition to allow for the production of UDP-galactose and UDP-*N*-acetylglucosamine (Table 2), the synthesis lacto-*N*-triose (LNT II), and transfer of the galactose moiety on UDP-galactose to LNT II, respectively. The UDP-galactose-4-epimerase and UTP-glucose-1-phosphate uridyltransferase DNAs were amplified from *E. coli* K12 genomic DNA and the glutamine fructose 6-phosphate aminotransferase, β-1,3-*N*-acetylglucosaminyltransferase, and β-1,3-galactosyltransferase DNAs were synthesized de novo. Additionally, two antibiotic resistance genes encoding the *Streptoalloteichus hindustanus* bleomycin resistance protein and *Tn5 E. coli* K12 neomycin phosphotransferase II were synthesized de novo and integrated along with the genes from *E. coli* K12, *Neisseria meningitidis*, and *Salmonella enterica* to facilitate selection of the recombinants.

The recombinants were then subjected to nitrosoguanidine (NTG) mutagenesis and screened for their ability to produce high levels of LNT, resulting in *JBT-LNT*. All gene insertions were verified by PCR using oligonucleotides specific to the coding sequence. Loss of the plasmids used to express the transposase, which contained an ampicillin resistance gene and temperature-sensitive origin of replication, was confirmed by growth at 42°C, ampicillin sensitivity, and failure to amplify plasmid specific DNA. All integrated genes remain in the genome. Although *JBT-LNT* possesses the neomycin phosphotransferase II, dihydrofolate reductase and bleomycin resistance genes used for integrant selection, no plasmids or other episomal vectors remain in the genome.

Table 2. Genetic Manipulations in *JBT-LNT*

Gene Product Name	Origin of the Gene	Manipulation	Effect
UDP-galactose-4-epimerase	*E. coli* K12	Ectopic expression	To enhance UDP-galactose synthesis
UTP-glucose-1-phosphate uridyltransferase	*E. coli* K12	Ectopic expression	
β-1,3-*N*-acetylglucosaminyltransferase	*Neisseria meningitidis*	Ectopic expression	To allow for the synthesis of lactose and lacto-*N*-triose (LNTII)
β-1,3-galactosyltransferase	*Salmonella enterica*	Ectopic expression	Catalyse the linkage of galactose derived from UDP-galactose on LNT II.
Glutamine fructose 6-phosphate aminotransferase	*E. coli* K12	Ectopic expression	To allow for the production of D-glucosamine 6-phosphate
Antibiotic resistance genes			
Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	Ectopic expression	To allow for the selection of recombinants during genetic engineering
Neomycin phosphotransferase II conferring resistance to kanamycin	*Tn5 E. coli* K12	Ectopic expression	

2. Manufacturing

a. Quality

Lacto-*N*-tetraose production occurs at the Jennewein Biotechnologie GmbH production facility in Maarweg 32, 53619 Rheinbreitbach, Germany, which is Food Safety System Certification (FSSC) 22000 and ISO 9001:2015 compliant, and a FDA-registered Food Facility (. Production also occurs at other Jennewein-qualified manufacturers that are GMP-, ISO-, and International Featured Standards Food 6.1-compliant via third party audits.

b. Processing Aids and Food Contact Substances

All raw materials, processing aids, and food contact substances used to produce the LNT powder are the same as those used to produce the 2'-FL that is the subject of GRN 571, which received a "no questions" letter from FDA. Therefore, the quality of the processing aids and raw materials and composition of the media described in GRN 571 (pg. 17; Appendix E, pg. 99-144; Appendix J, pg. 280-281) are incorporated by reference. Additional processing aids comply with European Pharmacopoeia, United States Pharmacopeia-National Formulary (USP-NF), or Japanese Pharmacopoeia specifications or appropriate product monographs. The water used throughout the manufacturing process complies with the TrinkwV, 2001 in Germany and the Council Directive 98/83/EC in the European Union and is non-fluoridated drinking water quality. All food contact surfaces (fermentation vessels and packaging materials) are either stainless steel or comply with the conditions of use that are specified in the US Code of Federal Regulations. The final product is packaged in food grade paper/low-density polyethylene (LDPE) bags in compliance with 21 CFR §177.1520. None of the processing aids are recycled or reused.

c. Production

Except for certain process parameters, LNT is manufactured using the same process as the 2'-fucosyllactose that is the subject of GRN 571. A detailed summary of the production process is provided in GRN 571 and is therefore incorporated by reference (see Section 2.2.2 of GRN 571). Briefly, the LNT production involves three steps (Figure 1). During Step 1, a carbon source and the substrate lactose are fermented by *JBT-LNT*, resulting in the production and secretion of the oligosaccharide into the culture medium. Lactase may be added at the end of the fermentation process to degrade excess lactose in the medium. Step 2 involves purification of the LNT from the culture medium. Step 3 involves spray-drying the LNT concentrate into a powder.

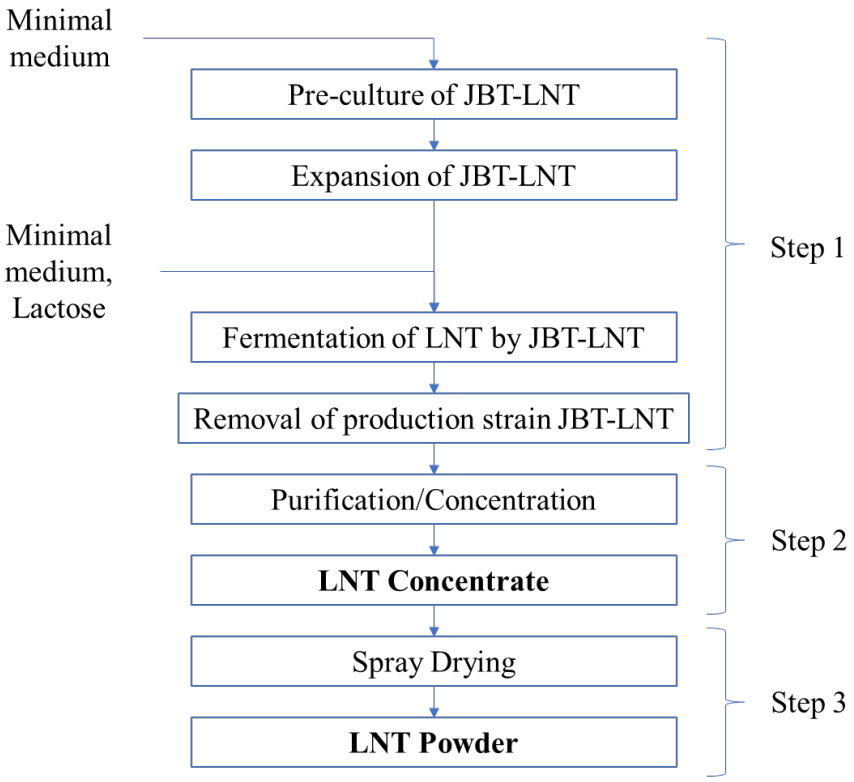


Figure 1. Production Process for Lacto-*N*-tetraose
JBT-LNT is expanded minimal medium and lactose to generates LNT. *JBT-LNT* is then removed yielding LNT-containing fermentation medium. The medium is purified, concentrated in a series of filtration, ion exchange, electrodialysis, and decolorization steps, and dried producing a powder containing LNT.

G. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. LNT Product Specifications and Batch Data

To ensure a consistent genetically modified-free, food-grade product, each batch of LNT is evaluated against a set of product specifications, which control the amount of LNT, carbohydrate by-products, DNA and endotoxin residues derived from the production strain, heavy metals, and selected microbes (Table 3). Each parameter is measured using either compendial or internally validated methods. The DNA testing method (GMO residues) is specific to the antibiotic resistance genes that were integrated into the genome of the production organism, and therefore serve as markers for production organism DNA contamination.

Data from three non-consecutive batches of LNT show that the manufacturing process reproducibly produces a finished product that complies with the product specifications and removes the production organism from the finished product.

Table 3. Product Specifications and Batch Data for Lacto-*N*-tetraose

Parameter	Analytical method	Specification	Batch Number		
			16125019	11043019	11047029
Physical Parameters					
Appearance (Color)[4]	visual	White to ivory-colored	Complies	Complies	Complies
Appearance (Form)[4]		Spray-dried powder	Complies	Complies	Complies
Chemical Parameters					
Lacto-*N*-Tetraose[4]	HPAEC-PAD	≥ 75 % DW (w/w)	84.2	81.8	81.6
Sum of Other carbohydrates[4]		≤ 25 % (% Area)	4.5	3.9	4.4
Lactose[4]		≤ 5 % (% Area)	1.8	0.3	0.4
Lacto-*N*-triose II[4]		≤ 5 % (% Area)	2.4	2.5	2.5
para-Lacto-*N*-Hexose[4]		≤ 5 % (% Area)	1.1	1.6	1.7
Glucose/galactose[4]		≤ 5 % (% Area)	< LOQ	1.1	1.1
Protein[4]	Nanoquant (modified Bradford)	≤ 100 µg/g	< LOQ	< LOQ	< LOQ
Ash[1]	ASU L 06.00-4	≤ 1 % (w/w)	0.44	0.23	0.34
Moisture[4]	KF titration	≤ 9% (w/w)	5.5	5.4	5.5
Endotoxins[3]	Ph. Eur. 2.6.14	≤ 10 EU/mg	0.228	0.034	0.031
Aflatoxin M1[1]	DIN EN ISO 14501	≤ 0.25 µg/kg	< LOQ	< LOQ	< LOQ
GMO residues[2]	qPCR	Negative	Negative	Negative	Negative
Heavy Metals					
Arsenic[1]	ASU L 00.00-135 – ICP-MS	≤ 0.2 mg/kg	ND	ND	ND
Cadmium[1]		≤ 0.1 mg/kg	ND	ND	ND
Lead[1]		≤ 0.02 mg/kg	ND	ND	0.016
Mercury[1]		≤ 0.5 mg/kg	ND	ND	ND
Microbes					
Standard Plate Count[1]	ISO 4833-2	≤ 10000 cfu/g	< 10	< 10	20
Yeast and Mold[1]	ISO 21527-2	≤ 100 cfu/g	< 20	< 20	< 20
Enterobacteriaceae[1]	ISO 21528-1	≤ 10 cfu/g	< 10	< 10	< 10
Salmonella [1]	ISO 6579	Absent/25 g	Absent	Absent	Absent
Cronobactor sakazakii[1]	ISO/TS 22964	Absent/10g	Absent	Absent	Absent

Abbreviations: DW, dry weight; cfu, colony forming units; STDEV, standard deviation; KF, Karl-Fischer; HPAEC-PAD, high performance anion exchange chromatography coupled with pulsed amperometric detection; qPCR, quantitative polymerase chain reaction; ICP-MS, inductively coupled plasma mass spectrometry; EU, endotoxin unit; Ph Eur., European Pharmacopoeia; LOQ, limit of quantitation.

[1]Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory; Ash LOQ = 0.01 %. Arsenic limit of detection (LOD) = 0.05 mg/kg; Cadmium LOD = 0.01 mg/kg; Mercury LOD = 0.005 mg/kg; Lead LOD = 0.01 ppm; Aflatoxin M1 LOQ = 0.025 µg/kg.

[2]Determined by GeneCon International GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory. Limit of detection = 0.01% of the finished product.

[3]Determined by Mikrobiologisches Labor. Dr. Michael Lohmeyer GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory; limit of quantitation = 0.005 EU/mg.

[4]Determined by Jennewein Biotechnologie using internally validated methods. Protein LOQ = 10 µg/g; carbohydrate by-products with a percent area greater than 0.5% (limit of quantitation) are considered.

2. Other Quality Attributes

a. Elemental Analyses

Although the LNT-containing medium produced during *JBT-LNT* fermentation is subjected to ion exchange chromatography and electrodialysis to remove the trace elements in the finished product, Jennewein quantitated the levels of manganese, selenium, iron, copper, molybdenum, nickel, zinc, and cobalt in six batches of the finished product (Table 4). Manganese and molybdenum were consistently below the limit of detection. Although cobalt was detected in three batches, the amount consumed by a one- and six-month-old infant from the proposed use level in infant formula will be 0.09 and 0.005 µg/kg/day (assuming that the average body weight of a one- and six-month old infant is 3.3 and 7.61 kg, respectively), which is below the provisional reference dose of 0.3µg/kg/day established by the Environmental Protection Agency (EPA) (US EPA, 2008). To ensure that the manufacturing process continues to produce a high-quality finish ingredient, these analyses will be conducted on an annual basis.

Table 4. Elemental Analysis of the Lacto-*N*-Tetraose

Element[1]	Method	Batch Number					
		1615019	11043019	11047029	21005010	21005020	21005030
Manganese (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7
Selenium (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.30	0.34	0.28	0.05	0.04	0.05
Iron (mg/kg)	ASU L 00.00-135 (ICP-MS)	1.5	1.2	1.4	1.9	1.6	1.8
Copper (mg/kg)	ASU L 00.00-135 (ICP-MS)	1.8	2.7	1.3	< 0.6	< 0.6	1.0
Molybdenum (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06
Nickel (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.10	0.11	0.11	0.32	0.33	0.34
Zinc (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.85	1.6	2.9	2.4	1.9	1.7
Cobalt (mg/kg)	PV-347 ICP-MS	0.22	0.22	0.23	< 0.04	< 0.04	< 0.04

[1]Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025 accredited laboratory; manganese limit of quantitation (LOQ) = 1.7 mg/kg; copper LOQ 0.6 mg/kg; molybdenum LOQ = 0.06 mg/kg; Cobalt LOQ = 0.04 mg/kg.

H. STABILITY OF HMO MIX

1. Genetic stability of *JBT-LNT*

To ensure genomic stability and finished product batch-to-batch consistency, all genes were introduced into the genome of the production strain *JBT-LNT* by either homologous recombination or transposition. Therefore, the strain does not harbor plasmids or episomal vectors. Thus, the production strain is not expected to lose its ability to produce a consistent finished product.

2. Stability of the LNT

The shelf-life of LNT is supported by ambient (25°C and 60% relative humidity) and accelerated (40°C and 75% relative humidity) stability studies conducted with the LNT that is the subject of GRN 833. Ambient (25°C and 60% relative humidity) and accelerated (40°C and 75% relative humidity) stability studies have also been conducted on a mixture of HMOs containing Jennewein's LNT. Because the Jennewein's LNT is qualitatively comparable and quantitatively similar to the subject of GRN 833 (see Table 9), it is reasonable to expect that the stability of Jennewein's will be similar to the LNT that is the subject of GRN 833. Therefore, the ambient and accelerated stability studies presented in GRN 833 are incorporated by reference and briefly summarized below, along with the ambient and accelerated stability studies conducted on the LNT-containing mixture of HMOs.

In the ambient stability studies conducted on the LNT that is the subject of GRN 833, chemical, physical, microbiological, and sensory testing was conducted on 2 representative batches of the finished ingredient over the course of 36 months (see pg. 78 of GRN 833). For one batch a complete set of chemical (moisture, LNT, lactose, lactulose, lacto-*N*-triose II, para-lacto-*N*-hexaose-2, LNT fructose isomer and human-identical milk saccharides), physical (color and appearance), and microbiological (aerobic mesophilic total plate count, Enterobacteriaceae, *Salmonella*, *Enterobacter sakazakii*, *Listeria monocytogenes*, *Bacillus cereus*, Yeasts, and Molds) data was available at 36 months and shows that all parameters tested complied with the product specifications. For the other batch, physical, chemical, and microbiological data was available for only 12 months and shows that results for all parameters tested, complied with the product specifications. Thus, it is reasonable to conclude that ingredient will be stabile when stored under ambient conditions for at least 36 months.

In the accelerated stability studies conducted on the LNT that is the subject of GRN 833, chemical, physical, microbiological, and sensory testing was conducted on one representative batch of finished ingredient over the course of 12 months (see pg. 81 of GRN 833). The results show that there are no changes in the organoleptic properties of LNT, no appreciable degradation of LNT, no changes in the impurity profile, and no alterations in the microbiological quality of the ingredient following storage over the course of 12 months.

To understand the stability of Jennewein's LNT when combined with other HMOs, a mixture containing 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 3'-sialyllactose (3'-SL), 6'-sialyllactose (6'-SL) and 23% LNT was stored in the high-density polyethylene bottles under ambient (25°C and 60% relative humidity) and accelerated (40°C and 75% relative humidity) conditions for 52 and 26 weeks, respectively. Lacto-*N*-tetraose and moisture content were monitored overtime using the same methods that are used for batch qualification.

Although there was an 8% reduction in LNT content by week 26 under ambient conditions, LNT content remained relatively unchanged over the course of the 52-week storage period. Because LNT content at week 39 and 52 were comparable to week 13, the 8% reduction at week 26 was likely due to analytical variability. Moisture content increased from 5.7 to 7.8 % over the course of the 52-week testing period (Table 5).

Under accelerated conditions, LNT steadily decreased, and moisture content increased over the course of 26 weeks (Table 6). Because no degradants were detected at week 52, the reduction in LNT content is likely due to analytical variation.

Because the stability studies conducted on the LNT that is the subject of GRN 833 shows that the ingredient is stabile for up to 36 months under ambient conditions, and the corroborating stability studies show that Jennewein's LNT is stabile when combined with other HMOs for up to 1 year under ambient conditions (EFSA NDA Panel, 2019; GRN 571; GRN 547; GRN 659; GRN 833), it is reasonable to expect that the Jennewein's LNT will have a comparable stability profile to the subject of GRN 833. Thus, these results support a shelf-life for Jennewein's LNT of 2 years from the date of production when stored under ambient conditions.

Table 5. Stability of Lacto-*N*-Tetraose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Ambient Conditions (25°C, 60% Relative Humidity)

Batch 4011-1004303107		**Moisture**		**LNT**	
		% DW	**% of baseline**	**% DW**	**% of baseline**
Interval	Baseline	5.7	100.0	23.61	100
	Week 1	5.2	91.9	23.70	100.4
	Week 4	6.2	109.2	23.45	99.3
	Week 8	6.1	108.3	23.40	99.1
	Week 13	6.1	107.2	23.40	99.1
	Week 26	6.9	121.7	21.65	91.7
	Week 39	7.3	129.3	23.25	98.4
	Week 52	7.8	137.0	23.05	97.6

Abbreviations: DW, dry weight; LNT, lacto-*N*-tetraose; NA, not applicable.

Table 6. Stability of Lacto-*N*-Tetraose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Accelerated Conditions (40°C, 75% Relative Humidity)

Batch 4011-1004303107		Moisture		LNT	
		% DW	% of baseline	% DW	% of baseline
Interval	Baseline	5.7	100.0	23.61	100
	Week 1	5.8	101.4	24.55	100.4
	Week 4	6.6	117.1	23.50	99.5
	Week 8	7.3	129.1	23.10	97.8
	Week 13	8.7	153.6	23.60	99.9
	Week 26	9.9	174.6	20.30	86.0

Abbreviations: DW, dry weight; LNT, lacto-*N*-tetraose.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

The intended effect of adding LNT to non-exempt term infant formula is to increase LNT intake in formula-fed infants and promote the growth of beneficial bacteria, including, but not limited to Bifidobacteria.

B. HISTORY OF EXPOSURE

LNT is one of the most abundant oligosaccharides in human milk. Synthetic forms of LNT have also been determined GRAS for use in infant formula and selected conventional foods (GRN 833). Thus, humans are exposed to LNT either through the ingestion of breast milk and/or products containing synthetic forms LNT.

The concentration of LNT in human breast milk has been quantitated in 30 studies with greater than 5 donors (Thurl et al., 1997; Thurl et al., 2010; Galeotti et al., 2012; Erney et al., 2000; Coppa et al., 1999; Sumiyoshi et al., 2003; Asakuma et al., 2008; Leo et al., 2010; Gabrielli et al., 2011; Bao et al., 2013; Nakhla et al., 1999; Chaturvedi et al., 1997; Chaturvedi et al., 2001; Asakuma et al., 2011). The results of 11 of these studies were summarized in a systematic review conducted by Thurl et al. (2017). A summary of the findings reported by Thurl et al. (2017) and the 17 additional studies is presented in Table 7. Although the levels of LNT in human milk vary with ethnicity, Secretor and Lewis-blood group status, lactation period, and term vs preterm birth, the available studies show that the concentration of LNT in breast milk generally ranges from 0.003 to 6.7 g/L with means and medians ranging from 0.1 to 3.9 and 0.2 to 2.1 g/L, respectively.

Additionally, a synthetic form of LNT is GRAS at levels up to 0.8 g/L in non-exempt, cow's milk-based infant formula for term infants; 0.6 g/L in drinks for young children (including toddler formulas); 5 g/kg in foods for infants and young children (including toddler foods); 10 g/kg in yogurt; 1 g/L in fluid milk (flavored and unflavored); 2 g/L in meal replacement drinks; 20 g/L in meal replacement bars; 10 g/kg in cereal and granola bars; and 1 g/L in soft drinks, fruit-based -ades, sports drinks, energy drinks, and enhanced waters (GRN 833, 2019).

Table 7. Studies Determining the Concentration of Lacto-*N*-tetraose (LNT) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	LNT concentration
Alderete et al., 2015	United States	37 donors	1 and 6 months	Highest median ± interquartile range: 1.4 ± 1.3 g/L (1 month) Lowest median ± interquartile range: 1.2 ± 1.0 g/L (6 month) *Only median ± interquartile ranges were reported
Austin et al., 2016	China	450 donors (approximately 90 donors/timepoint)	Days 5-11, Days 12-30, Months 1-2, Months 2-4, Months 4-8	Reported range: 0.02 – 3.0 g/L Highest mean: 0.9 ± 0.5 g/L (days 5-11) Highest median: 0.8 g/L (days 5-11) Lowest mean: 0.3 ± 0.2 g/L (4-8 months) Lowest median: 0.2 g/L (4-8 months)
Austin et al., 2019	Switzerland	27 donors with 33 preterm infants (approx. 25 samples/timepoint) 34 donors with 34 term infants (approx. 28 samples/timepoint)	Weekly for 8 weeks after delivery (preterm and term) then every 2 weeks until 16 weeks (preterm only)	Reported range: 0.14 – 3.8 g/L Highest mean: 1.5 ± 0.7 g/L (Preterm, week 2) Highest median: 1.3 g/L (Term, week 3) Lowest mean: 0.7 ± 0.4 g/L (Preterm weeks 14 and 16) Lowest median: 0.6 g/L (Preterm week 14)
Azad et al., 2018	Canada	427 donors	3- 4 months postpartum	Reported range: 0.1 – 5.6 g/L Mean: 1.5 ± 0.7 g/L Median: 1.4 g/L
Chaturvedi et al., 1997	Mexico	50 donors	1-2 months postpartum	Mean: 0.2 ± 0.009 g/L *Only mean ± standard error was reported
Kunz et al., 2017	Spain	32 donors (21 secretors; 11 nonsecretors	Lactation days 1-7 (colostrum), 8-15 (transitional milk), and 16-30 (mature milk)	Highest median: 1.0 g/L (0.6 – 2.8 g/L) (Preterm, mature milk) Lowest median: 0.8 g/L (0.6-1.6 g/L) (Term, Colostrum) *Only median and interquartile ranges were reported

Table 7. Studies Determining the Concentration of Lacto-*N*-tetraose (LNT) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	LNT concentration
Larsson et al., 2019	Denmark	11 mothers with high weight infants 15 mothers with normal weight infants	5 and 9 months	Highest median: 1.0 g/L (0.8 – 1.1 g/L) (5 months; normal weight group) Lowest median: 0.6 g/L (0.3 – 0.5 g/L) (9 months; high weight group
Leo et al., 2010	Samoa	8 mothers	5-10 days and greater than 10 days postpartum	Highest mean: 3.9 ± 1.9 g/L (5-10 days post-partum) Lowest mean: 1.3 ± 0.6 g/L (greater than 10 days post-partum) *Median and range was not reported
Ma et al., 2018	China, Malaysia	China: 20 donors Malaysia: 26 donors	China: days 14, 30, 60, 90, 120, 180, and 240 post-partum Malaysia: days 2, 60, 180, and 365 post-partum	<u>Chinese Mothers</u> Highest mean: 2.0 ± 0.7 g/L (14 days post-partum) Lowest mean: 0.8 ± 0.5 g/L (240 days post-partum) <u>Malaysian Mothers</u> Highest mean: 2.4 ± 2.1 g/L (2 days post-partum) Lowest mean: 1.1 ± 0.6 g/L (365 days post-partum) *Only means ± standard deviations were reported
Marx et al., 2014	United States	26 mothers with infants in the neonatal intensive care unit 31 samples of donor milk	Random	<u>Mothers milk</u> Reported range: ~0.1 – 8.5 g/L Median (interquartile range): ~2.1 (1.6 - 3.9) g/L <u>Donor milk</u> Reported range: ~0.4 – 4.1 g/L Median and interquartile range: ~1.2 (0.6 - 2.0) g/L *values obtained from a graph

Table 7. Studies Determining the Concentration of Lacto-*N*-tetraose (LNT) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	LNT concentration
McGuire et al., 2017	Ghana, Kenya, Peru, Spain, Sweden, rural and urban Ethiopia and Gambia, Washington State (USA), and California (USA)	410 healthy women	2 weeks to 5 months postpartum	Highest mean: 1.6 ± 0.16 g/L (Gambia Rural; n=40) Lowest mean: 0.7 ± 0.1 g/L (Peru; n=43) *Only means ± standard deviations were reported
McJarrow et al., 2019	United Arab Emerites	Transitional milk: 41 donors Mature milk: 40 donors	Days 5-15 post-partum (transitional milk) 6 months post-partum (mature milk)	Highest mean: 1.4 ± 0.7 g/L (Transitional milk) Lowest mean: 0.5 ± 0.3 g/L (Mature milk) *Only means ± standard deviations were reported
Nijman et al., 2018	United States	10 donors	Day 3 and 42 postpartum	Highest mean: 0.5 ± 0.03 g/L (day 42) Lowest mean: 0.5 ± 0 g/L (day 3)
Paganini et al., 2019	Kenya	80 donors	No specific timepoint	Median (interquartile range): 0.5 (0.5-0.7) g/L *Mean and range was not reported
Samuel et al., 2019	Europe	290 donors	Days 2, 17, 30, 60, 90, and 120 of lactation	Reported range: 0.02 – 6.7 g/L Highest mean: 1.2 ± 0.7 g/L (day 17 postpartum) Highest median: 1.0 g/L (day 17 postpartum) Lowest mean: 0.5 ± 0.4 g/L (day 120 postpartum) Lowest median: 0.5 g/L (day 120 postpartum)
Sjogren et al., 2007	Sweden	11 allergic 9 non-allergic women	2-4 days postpartum	Range: 0.2 - 2.2 g/L Highest median: 0.8 g/L (non-allergic mothers) Lowest median: 0.6 g/L (allergic mothers) *Means were not reported
Spevacek et al., 2015	United States	Mothers of 15 term and 13 preterm	Colostrum (1st week), transition (14 days postpartum), and mature milk (28 d postpartum)	Highest mean: 1.3 ± 1.0 g/L (Preterm mature milk) Lowest mean: 0.7 ± 0.7 g/L (Preterm colostrum) *Medians were not reported

Table 7. Studies Determining the Concentration of Lacto-*N*-tetraose (LNT) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	LNT concentration
Sprenger et al., 2017	Singapore	Approx 50 donors	1, 2, and 4 months postpartum	Reported range: 0.003 – 2.8 g/L Highest mean: 1.5 ± 0.6 g/L (1 month postpartum) Highest median: 1.2 g/L (1 month postpartum) Lowest mean: 0.4 ± 0.2 g/L (4 months postpartum) Lowest median: 0.3 g/L (4 months postpartum)
Sumiyoshi et al., 2003	Japan	16 donors	4, 10, 30, and 100 days postpartum	Reported range: 0.003 – 1.0 g/L Highest mean: 0.4 ± 0.08 g/L (10 days postpartum) Lowest mean: 0.1 ± 0.04 g/L (100 days postpartum) *medians were not reported
Thurl et al., 2017	Worldwide	Systematic review of 21 previous studies (not all reported LNT)	Lactation days 0 to >100	Highest mean 1.04 g/L (95% confidence limit of 0.39 – 1.68; n=75 preterm mothers/356 samples) Lowest mean: 0.79 g/L (95% confidence limit of 0.59 – 0.98; n=117 term mothers/308 samples) *Medians were not reported
Williams et al., 2017	United States (Washington and Idaho)	16 donors	Weekly for 7 months (average time post-partum at enrollment 161 days)	Mean = 0.32 ± 0.044 g/L *Only one mean ± standard error was reported

C. INTENDED USES

Jennewein intends to use LNT powder as a substitute for other forms of LNT in non-exempt, cow's milk-based infant formula for term infants at a level of 1.82 g/L. Although this level is approximately 2.25-fold greater than what is GRAS (GRN 833, 2019), the results reported in the studies that quantitated the levels of LNT in breast milk indicate that the increased level of use will adequately accommodate variations in LNT levels due to ethnicity, Secretor and Lewis-blood group status, lactation period, and term vs preterm birth.

D. ESTIMATED DAILY INTAKE

Because Jennewein intends to use its LNT powder as a substitute for other forms of LNT currently marketed in the United States at a level approximately 2.25-fold greater than what is GRAS (GRN 833), the resulting mean and 90th percentile estimated daily intakes of LNT from infant formula in infants 0 to 6 months-of-age provided in GRN 833 will increase from 1.12 and 2.20 g/day (176 and 301 mg/kg bw/day) to 2.52 and 4.95 g/day (396 and 677 mg/kg bw/day), respectively, which represents the maximum level of intake because infants at this age consume infant formula as a sole source of nutrition.

IV. SELF-LIMITING LEVELS OF USE

This part does not apply.

V. COMMON USE IN FOOD BEFORE 1958

This part does not apply.

VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The general recognition of safety of LNT according to the specified conditions of use in non-exempt term infant formula is based on the following: the published studies that quantitate the levels of LNT in human milk (see Section III.B); the analytical data demonstrating that the LNT produced by Jennewein is structurally identical to LNT from human milk; the qualitative and quantitative similarities between the subject of this GRAS Notice and the LNT ingredient that is produced by Glycom A/S, was tested by in the genotoxicology and subchronic studies published by Phipps et al. (2018), and supports the GRAS status of the LNT that is the subject of GRN 833; published genotoxicology and subchronic toxicology studies conducted with LNT alone or LNT in combination with other HMOs; and a tolerance study in neonatal piglets.

Human milk is the reference standard for infant nutrition (Section on Breastfeeding, 2012). As the sole source of nutrition for breast-fed infants, human milk contains the essential nutrients for healthy growth and development (Section on Breastfeeding, 2012). Among its numerous components are non-digestible oligosaccharides, also known as human milk oligosaccharides (HMOs), which are one of the most prevalent solid components and believed to play to an important role in promoting the growth of the infant gastrointestinal tract microbiota and maturation of the intestinal mucosal immune system (Kunz et al., 1999; Jost et al., 2015). Structurally they contain glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and *N*-acetyl-neuraminic acid moieties (Neu5Ac) (Milani et al., 2017). All HMOs have lactose (Galβ1-4Glc) at the reducing end and elongated oligosaccharide chains composed of either lacto-*N*-biose (Galβ1-3GlcNAc) or *N*-acetyllactosamine (Galβ1-4GlcNAc) disaccharide units linked by β1-3 or β1-6 glycosidic bonds at the non-reducing end. A β1-6 glycosidic bond between two disaccharide units introduces chain branching. Additionally, lactose and the elongated oligosaccharide chains can be fucosylated via α1-2, α1-3, or α1-4 linkages or sialyllated via α2-3, or α2-6 linkages. Currently, more than 200 different HMOs have been identified and the highest levels of HMOs are found in colostrum (20-25 g/L) (Bode, 2012).

Non-fucosylated neutral HMOs constitute 42-55% of the total HMO fraction in breast milk and LNT is one of the more abundant non-fucosylated neutral HMOs (Van Niekerk et al., 2014; Smilowitz et al., 2014; Thurl et al., 2017). Although its absolute concentration varies with ethnicity, Secretor and Lewis-blood group status, and lactation period, LNT is highest in transitional milk (up to 3.9 g/L) and lowest in mature breast milk (0.1 g/L) (Erney et al., 2000). Additionally, published studies show that the concentration of LNT in breast milk generally ranges from 0.003 to 6.7 g/L with means and medians ranging from 0.1 to 3.9 and 0.2 to 2.1 g/L,

respectively. Thus, Jennewein's intended use of 1.82 g LNT/L infant formula is well within the established range of LNT that occurs naturally in breast milk.

Because human milk is the reference standard for infant nutrition, infant formula manufacturers look to mimic the composition and functionality of human milk in their formulas as much as possible. Manufacturing HMOs on a commercial scale, however, has not been feasible until recently and in its place, infant formula manufacturers have resorted to supplementing their formulas with other synthetic and plant-based non-digestible oligosaccharides to confer the prebiotic effects of HMOs. These other oligosaccharides include galactooligosaccharides (GOS), polydextrose, oligofructose, long-chain inulin, and fructooligosaccharides (FOS) (GRN 233, 2009; GRN 285, 2009; GRN 286, 2009; GRN 334, 2010; GRN 392, 2011; GRN 477, 2013; GRN 484, 2014; GRN 495, 2014; GRN 518, 2014; GRN 537, 2014; GRN 569, 2015; GRN 576, 2015; GRN 623, 2016; GRN 620, 2016; GRN 797, 2018). Galactooligosaccharides (GOS), specifically, are GRAS for use in infant formula at levels up to 7.2 g/L. Although their safe use is supported by extensive preclinical and clinical data, GOS and the other non-HMOs are simply not natural or innate components of breast milk.

Additionally, the use of selected HMOs opposed to a mixture of the almost 200 HMOs in infant formula has been called into question (Milani et al., 2017). However, it is important to note that breast milk is considered to be the reference standard for infant nutrition, both the types and amounts of HMOs in breast milk can vary greatly from one mother to another, and observational studies that investigated the effects of varying breast milk HMO composition on infant growth and health have reported conflicting results due to design limitations and/or confounding factors (Alderete et al., 2015; Azad et al., 2018; Berger et al., 2020; Lagström et al., 2020; Gridneva et al., 2019; Kuntz et al., 2019; Larsson et al., 2019; Sprenger et al., 2017; Vandenplas et al., 2018). Thus, a clear and consistent link between the use of selected and structurally different HMOs in infant formula and adverse outcomes on infant growth and health does not exist. Therefore, based on the totality of the available evidence, it is reasonable to expect that supplementing infant formula with a synthetic form of LNT will not pose risks to infants consuming formula containing LNT.

In the United States, one LNT ingredient is GRAS for use in non-exempt term infant formula, as well as a variety of food products (GRN 833). It is synthesized by fermentation and structurally identical to the LNT found in breast milk. Additionally, its use in infant formula and food products is supported by use levels that are within the range of LNT levels found in breast milk and a variety of published genotoxicity and subchronic toxicity studies conducted in neonatal rats with an LNT-containing ingredient produced by fermentation (Phipps et al., 2018). Importantly, because the LNT ingredient produced by Jennewein is structurally identical and

chemically equivalent to the LNT ingredient that was tested by Phipps et al. (2018) and the subject of GRN 833 and will be used as a substitute for the LNT that is GRAS, the published studies conducted by Phipps et al. and reviewed in GRN 833 support the safe use of Jennewein's LNT ingredient in infant formula.

The safe use of Jennewein's LNT ingredient is supported by a battery of published and unpublished genotoxicity, subchronic toxicity, and tolerability studies (Phipps et al., 2019; Parschat et al., 2020; unpublished neonatal piglet study). Because Jennewein's LNT is qualitatively comparable and quantitatively similar to LNT that was manufactured by Glycom A/S and tested by Phipps et al. (2018), the genotoxicity and subchronic toxicity studies published by Phipps et al. are the pivotal results that support safety of Jennewein's LNT. Lacto-*N*-tetraose is not genotoxic and has a NOAEL (no observed adverse effect level) of at least 4 g/kg bw/day, which was the highest dose tested (Phipps et al., 2018). Additional genotoxicity and subchronic toxicity studies conducted by Parschat et al. (2020) and an unpublished neonatal piglet study, which were conducted with a mixture of HMOs containing LNT manufactured by Jennewein, corroborate results reported by Phipps et al. (2018).

Based on these data, there is reasonable certainty that the use of Jennewein's LNT per the intended use and use level is of no harm to consumers. Jennewein's LNT is therefore GRAS as an ingredient in cow's milk-based, non-exempt, term infant formula at the intended use level.

A. SAFETY OF THE PRODUCTION ORGANISMS

The subject of this GRAS Notification is produced using *JBT-LNT*, which is derived from the host organism *E. coli* BL21(DE3). *E. coli* BL21(DE3) is routinely used in the industry for manufacturing food and pharmaceutical ingredients, and its safety is thoroughly summarized in GRN 485 (pg. 15-18), GRN 571 (Appendix K, pg. 282-300), and the GRN 571 Supplement, all of which received "no questions" letters from the FDA. GRN 485 and 571 describe the use of E. *coli* BL21(D3) as the host organism in the production of BbgIV beta-galactosidase and 2'-FL, respectively.

Escherichia coli are commensal residents of the gut microflora of humans and numerous animal species. *E. coli* strains are taxonomically grouped into five different phylogroups (A, B1, B2, D, and E) based on the sequence similarity of housekeeping genes (Archer et al., 2011). Human commensal strains are typically found in Group A or B1, with non-related pathogenic strains classified under Group B2, D, and E. Three group A laboratory strains as well as strains K-12, B, C, and their derivatives are designated as Risk Group 1 organisms according to their relative pathogenicity for healthy adult humans (Archer et al., 2011; Daegelen et al., 2009). Under current National Institutes for Health (NIH) guidelines for research involving

recombinant or synthetic nucleic acid molecules, Risk Group 1 organisms "are not associated with disease in healthy adult humans" (National Institutes of Health, 2019). Of these strains, *E. coli* K-12 and the B derivatives (*e.g.*, BL21) are among the most widely used for production of biotechnology products, and for production of industrial, pharmaceutical, and food biotechnology preparations.

Several comprehensive studies have demonstrated the safety of *E. coli* BL21(DE3). This strain does not carry the well-recognized pathogenic components required by *E. coli* strains that cause the majority of enteric infections. *E. coli* BL21(DE3) is therefore considered to be non-pathogenic and unlikely to survive in host tissues or to cause disease (Chart et al., 2000). *E. coli* BL21(DE3) was one of the first organisms to have its complete genome sequence assembled and differs only marginally from another widely used production strain, *E. coli* K-12 (Studier et al., 2009). This sequencing revealed the absence of genes encoding invasion factors, adhesion molecules, and enterotoxins associated with virulence (Jeong et al., 2009). Finally, an acute oral toxicity study showed that the *E. coli* BL21(DE3) endotoxin produced no toxicity in mice, even at the highest dose of 1,000,000 EU (3.3 mg/kg body weight) (Harper et al., 2011).

Based on the comprehensive characterization of this strain and its widespread use in protein production, safety issues resulting from the use of *E. coli* BL21(DE3) as a host strain are not expected. Additionally, because all genetic modifications in the production organism are chromosomally integrated, result in the expression of well-characterized proteins, and the organism is removed from the finished ingredient during manufacturing, there is reasonable certainty that neither the host organism nor the genetic modifications to *JBT-LNT* pose safety risks to the consumer.

B. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

The absorption, distribution, metabolism and excretion (ADME) of HMOs has been extensively summarized in previous GRAS Notices and opinions published by authoritative bodies around the world (GRN 484, 2014; GRN 546, 2015; GRN 547, 2014; GRN 571, 2015; GRN 650, 2016; GRN 659, 2016; GRN 735, 2018; GRN 749, 2018; GRN 766, 2018; GRN 815, 2019; GRN 833, 2019; EFSA Panel on Dietetic Products, 2015; EFSA Panel on Nutrition et al., 2019). Briefly, HMOs, including LNT, are highly resistant to the digestive enzymes of the gastrointestinal (GI) tract and only small amounts are absorbed intact. *In vitro* studies have shown that <5% of ingested HMOs is digested. *In vivo* studies among infants and in rats have reported that 1 to 2% of the total amount of ingested HMO is excreted unchanged in urine and the remaining unabsorbed oligosaccharides then pass through the gastrointestinal tract where it is either fermented by the select resident microbiota or excreted unchanged in the feces (Goehring

et al., 2014; Ruhaak et al., 2014; Santos-Fandila et al., 2014; Dotz et al., 2014; Obermeier et al., 1999; Rudloff et al., 2012; Rudloff et al., 2006; Rudloff and Kunz, 2012; Rudloff et al., 1996; Chaturvedi et al., 2001; Gnoth et al., 2000; Engfer et al., 2000; Brand-Miller et al., 1998). Although the exact mechanisms by which HMO absorption occurs have not been fully elucidated, data from *in vitro* studies using the Caco-2 human intestinal epithelial cell line suggest that neutral HMOs, such as LNT, are transported across the intestinal epithelium by receptor-mediated transcytosis as well as by paracellular transport, whereas acidic HMO are absorbed via the non-specific paracellular transport only (Gnoth et al., 2000).

C. TOXICOLOGY STUDIES

The pivotal genotoxicity and subchronic toxicity studies that support the safe use of Jennewein's LNT in infant formula are OECD 408-compliant bacterial reverse mutation test, an OECD 471-compliant in vitro micronucleus test, a 14-day range-finding oral toxicity test in neonatal rats, and an OECD 408-compliant 90-day oral toxicity test in neonatal rats, all of which were conducted using an ingredient that contained 77% LNT manufactured fermentation by Glycom A/S, published by Phipps et al. (2018), and used to support GRAS status of Glycom A/S's LNT ingredient (GRN 833). Additionally, corroborating genotoxicity and subchronic toxicity studies have been conducted with a mixture containing 2'-FL, 3'-FL, LNT, 3'-SL, 6'-SL, all of which are manufactured by Jennewein using carefully controlled fermentation conditions (Parschat et al., 2020).

Jennewein's LNT ingredient contains LNT and variety of by-products including *N*-acetylglucosamine, glucose, galactose, lacto-*N*-triose II (LNT II), lacto-*N*-biose, galactosylactose, glucosyllactose, and other lacto-*N*-biose- and *N*-acetylglucosamine-containing LNT by-products, such as para-lacto-*N*-hexose 2 (LNT-$(LNB)_1$) (Table 8). To determine whether or not the genotoxicity and subchronic toxicity studies conducted by Phipps et al. support the GRAS status of Jennewein's LNT ingredient, the carbohydrate profile of the LNT used by Phipps et al. (2018) and the product specifications for the LNT that is the subject of GRN 833 and this Notice were compared. The LNT used by Phipps et al. contained 77% LNT and 19.4% LNT II, para-lacto-*N*-hexose 2, an LNT fructose isomer, and other carbohydrates (Table 9). The subject of GRN 833 contains similar levels of LNT, LNT II, para-lacto-*N*-hexose 2, an LNT fructose isomer, and other carbohydrates based on the product specifications listed in GRN 833. Additionally, the subject of GRN 833 also contains small amounts of lactulose, lactitol, glucose, galactose, fructose, ribose, a reduced form of LNT (Gal-GlcNAc-Gal-sorbitol), isomaltose, GlcNAc-LNT and 3-Gal-LNT, all of which are controlled by a "sum of other carbohydrates" product specification. Importantly, for Jennewein's LNT, product specifications are in place that result in levels of LNT, lactose, and LNT II that are comparable to both the ingredients tested by

Phipps et al. and subject of GRN 833. Specifications are also in place that control the levels of glucose and galactose, and the LNnT fructose isomer and lactulose present in the subject of GRN 833 are not expected in Jennewein's LNT ingredient due to the manufacturing process. Furthermore, the LNT, LNT II, lactose, ash, and water product specifications for both Jennewein's and Glycom's LNT ingredients account for not less than 95% of product on a weight/weight basis. Thus, the amount of the by-products *N*-acetylglucosamine, lacto-*N*-biose, galactosylactose, glucosyllactose, and other lacto-*N*-biose- and *N*-acetylglucosamine-containing LNT by-products in Jennewein's LNT ingredient is limited to only minute quantities, and significant differences in the quality and safety among Jennewein's LNT, the LNT tested by Phipps et al. and the LNT that is the subject of GRN 833 are not expected.

Table 8. Carbohydrate By-Product Profile of Jennewein Lacto-*N*-tetraose[1]

Carbohydrate By-product	Batch Number		
	10916019	10916029	10916039
N-Acetylglucosamine (% area)	1.0	1.0	1.1
Lacto-N-biose (% area)	0.6	0.7	0.6
Galactosyllactose (% area)	< 0.5	< 0.5	< 0.5
Glucosyllactose (% area)	1.0	1.3	0.8
LNT-$(LNB)_n$ (% area)[2]	3.0	2.3	2.4
LNT-$(LNB)_n$-GlcNAc (% area)	0.8	1.3	0.8
Total unspecific impurities by HPLC	3.3	4.0	3.8

Gal, galactose; Glc, glucose; LNT, lacto-*N*-tetraose; LNB, lacto-*N*-tetraose byproduct; n, repeating unit
[1]Determined by Jennewein Biotechnologie using HPAEC-PAD, high performance anion exchange chromatography coupled with pulsed amperometric detection. Carbohydrate by-products with a percent area greater than 0.5% (limit of quantitation) are considered.
[2]Includes para-lacto-N-hexose2

Table 9. Comparison of Jennewein's LNT with the LNT Tested by Phipps et al., 2018 and That is the Subject of GRN 833

Parameter	GRN 833		Jennewein's Specifications
	Phipps et al., 2018	Specifications	
Lacto-*N*-Tetraose	77% DW	≥ 70 % DW	≥ 75 % DW
Lactose	8.9%	≤ 12 w/w %	≤ 5 % (% Area)
Lacto-*N*-triose II	6.0%	≤ 10 w/w %	≤ 5 % (% Area)
Para-Lacto-*N*-Hexose	NP	NS	≤ 5 % (% Area)
Para-Lacto-*N*-Hexose2	2.5%	≤ 3.5 w/w %	NS
Glucose/galactose	NP	NS	≤ 5 % (% Area)
LNT fructose isomer	0.6%	≤ 1 w/w %	NS
Others	1.4%	≤ 5 w/w %[2]	NS[3]
Moisture	2.0 %	≤ 6 w/w %[2]	≤ 9% (w/w)
Ash	0.1%	≤ 0.5 w/w %	≤ 1 % (w/w)

Abbreviations: DW, dry weight; NS, not specified; NP, not provided; HiMS, human identical milk saccharides
[1]Includes lactose, lacto-N-triose II, para-lacto-N-hexose, glucose/galactose, as well as all of the other minor by-products detected with HPAEC-PAD (high performance anion exchange chromatography coupled with pulsed amperometric detection)
[2]As summarized in GRN 833, the sum of other carbohydrates includes lactulose, lactitol, glucose, galactose, fructose, ribose, the reduced form of LNT ("Gal-GlcNAc-Gal-sorbitol"), isomaltose, lacto-N-triose II fructose isomer ("GlcNAc-Gal-fructose"), GlcNAc-LNT and 3-Gal-LNT. The main contribution derives from the reduced carbohydrates (lactitol and reduced form of LNT) and isomaltose.
[3]Includes lactose, lacto-N-triose II, para-lacto-N-hexose2, LNT fructose isomer, and others.

Therefore, because Jennewein's and Glycom's LNT ingredients contain LNT that is structurally identical to the LNT in breast milk and are qualitatively comparable and quantitatively similar, the toxicity studies published by Phipps et al. (2018), which support the safe use of the subject GRN 833, also support the safe use of Jennewein's LNT in infant formula. The genotoxicity and subchronic toxicity studies conducted by Phipps et al. (2018), which are extensively summarized in GRN 833, are therefore incorporated by reference and briefly summarized below. Additionally, the OECD-compliant bacterial reverse mutation assay, OECD-compliant in vitro micronucleus assay, seven-day pilot dietary toxicity study and OECD-compliant 90-day feeding study conducted by Parschat et al. (2020) are also summarized below. In summary, LNT is not genotoxic in the presence or absence of other HMOs and has an no observed adverse effect level as a single ingredient of 4000 mg/kg bw/day in neonatal rats.

1. Genotoxicity Studies

a. Studies on Lacto-N-Tetraose as a Single Ingredient

i. Bacterial Reverse Mutation Assay

As summarized on page 38 of GRN 833, Phipps et al. (2018) evaluated the mutagenicity of a LNT powder manufactured by Glycom A/S containing 77% LNT in an OECD-471-

compliant bacterial reverse mutation test (Ames test). Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella Typhimurium* strains TA98, TA100, TA1535 and TA1537 and the *Escherichia coli* strain WP2 uvrA (pKM101). All strains were treated with LNT at concentrations of up to 5,106.1 μg/plate, which exceeded OECD's recommended maximum concentration due to a minor error in the application of the correction factor, in the absence and presence of external metabolic activation (S9 mix). Water (purified by reverse osmosis) served as the vehicle for LNT and as the negative control. Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide) and presence (2-aminoanthracene and benzo(a)pyrene) of metabolic activation. A positive result for mutagenicity was defined as a dose-dependent and biologically relevant 2-or 3-fold increase in the number of revertant colonies, compared to that of the vehicle control group. There was no evidence of mutagenicity following exposure to LNT in either test, in the absence or presence of metabolic activation, indicating that LNT is not mutagenic up to 5,106.1 μg/plate.

ii. *In vitro* Micronucleus Assay

As summarized in on pg. 39, Phipps et al. (2018) evaluated the clastogenicity and aneurgenicity of the LNT powder manufactured by Glycom A/S containing 77% LNT in an OECD-487-compliant in vitro micronucleus test. Human lymphocytes were treated with concentrations of LNT at 510.61, 1,021.22 or 2,042.44 μg/ml in the presence or absence of metabolic activation. Due to a minor error in the application of a correction factor, the highest dose exceeded OECD's recommended maximum concentration. The negative control was water and the positive controls were colchicine, mitomycin C, and cyclophosphamide in the presence and absence of metabolic activation. No cytotoxicity was observed up to 2042.44 μg/ml in the presence or absence of metabolic activation. There was no evidence of clastogenicity or aneugenicity in any of the tests, both in the absence or presence of metabolic activation, indicating that LNT is not clastogenic or aneugenic at concentration up to 2042.44 μg/ml.

b. Studies on Jennewein's LNT as Part of HMO Mixture

i. Bacterial Reverse Mutation Test

To evaluate the mutagenicity of an HMO mixture containing 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, Parschat et al. (2020) conducted an OECD-complaint bacterial reverse mutation test. Five strains of S. typhimurium (TA98, TA100, TA102, TA1535, and TA1537) were used in two independent experiments with and without metabolic activation. The first experiment was conducted as a

plate incorporation test and the second as a preincubation test (Ames et al., 1973; Ames et al, 1975; Maron and Ames, 1983). Five, 10.0, 31.6, 100, 316 or 600 mg of the mixture containing 1.2, 2.4, 7.5, 23.7, 74.9, and 142.2 mg LNT, respectively, were applied to each plate. Purified water was the negative control and the positive controls for the different strains were sodium azide (for TA1535 and TA100), 2-nitrofluorene (for TA98), benozo[a]pyrene 9AA (for TA1537), and mitomycin C (for TA102). Cytotoxicity was defined as a reproducible reduction in the number of colonies by more than 50% compared to the solvent control and/or a scarce background lawn.

Compared to the negative control, the positive controls increased the mean revertant colony numbers at least threefold with and without metabolic activation (Table 9), verifying the validity of the test. For the HMO mixture, no cytotoxicity or mutagenicity were noted in any of test strains up to 600 mg HMO mixture/plate (equivalent to 142.2 mg LNT/plate) in either the plate incorporation or preincubation tests (Table 10). Parschat et al. concluded the HMO mixture, and the LNT contained therein, was not mutagenic under the conditions tested.

Table 10. Bacterial Reverse Mutation Test Performed with an HMO Mixture Containing 23.7% Lacto-*N*-Tetraose[c]

HMO Mixture (mg/plate)	Number of revertant colonies per plate									
	TA98		TA100		TA102		TA1535		TA1537	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Plate incorporation test										
Negative control (water)	26.3 ± 4.2	25.3 ± 3.2	153.7 ± 28.3	151.7 ± 6.8	287.0 ± 13.0	276.7 ± 26.7	17.0 ± 3.6	17.0 ± 2.6	5.3 ± 0.6	9.3 ± 0.6
5	28.3 ± 2.9	31.0 ± 5.2	139.3 ± 3.2	167.7 ± 15.5	252.0 ± 4.6	274.3 ± 15.5	15.7 ± 4.6	21.7 ± 1.5	5.3 ± 2.5	8.0 ± 1.7
10	29.0 ± 1.0	32.3 ± 6.7	129.3 ± 10.1	159.0 ± 19.1	273.3 ± 2.9	256.7 ± 13.1	16.0 ± 1.0	18.0 ± 4.4	5.0 ± 0.0	7.7 ± 0.6
31.6	28.0 ± 2.0	31.0 ± 8.2	129.3 ± 3.8	160.0 ± 7.8	283.7 ± 37.4	266.3 ± 2.5	15.0 ± 1.0	14.3 ± 2.5	6.7 ± 3.2	5.7 ± 0.6
100	29.0 ± 3.0	31.0 ± 10.0	158.7 ± 12.0	162.7 ± 24.2	278.3 ± 18.8	256.7 ± 9.7	15.7 ± 1.2	16.3 ± 2.1	7.0 ± 2.6	7.3 ± 1.2
316	26.0 ± 1.0	27.0 ± 8.2	145.3 ± 12.6	172.7 ± 6.4	264.3 ± 3.8	254.7 ± 9.8	15.0 ± 1.7	18.7 ± 4.0	7.0 ± 1.7	5.7 ± 1.2
600	24.7 ± 2.5	26.3 ± 2.1	157.0 ± 35.5	177.0 ± 4.4	252.7 ± 1.2	274.3 ± 1.2	15.7 ± 2.3	16.7 ± 3.1	6.0 ± 0.0	7.0 ± 3.0
Positive control[a,b]	179.7 ± 15.3	175.7 ± 28.7	892.0 ± 13.9	887.3 ± 11.6	918.3 ± 34.8	911.7 ± 18.1	147.0 ± 19.1	158.7 ± 27.2	73.3 ± 4.0	74.3 ± 3.2
Preincubation test										
Negative control (water)	29.7 ± 1.5	37.3 ± 1.5	182.0 ± 6.2	164.7 ± 35.3	285.3 ± 1.5	283.3 ± 8.4	22.7 ± 7.8	17.0 ± 2.6	6.7 ± 2.3	6.0 ± 2.6
5	33.3 ± 8.3	25.3 ± 2.5	165.0 ± 3.6	155.7 ± 4.9	283.3 ± 7.2	273.3 ± 10.3	14.7 ± 2.1	21.3 ± 1.5	7.0 ± 0.0	6.7 ± 3.5
10	32.7 ± 2.5	28.7 ± 6.4	169.3 ± 12.7	171.3 ± 10.8	295.7 ± 7.1	277.7 ± 18.6	16.3 ± 2.3	16.0 ± 3.6	6.0 ± 2.0	5.3 ± 2.3
31.6	26.7 ± 4.7	30.7 ± 4.0	171.0 ± 12.8	158.7 ± 23.1	301.3 ± 13.3	298.3 ± 5.5	17.7 ± 2.3	16.0 ± 4.4	8.3 ± 2.1	4.3 ± 1.2
100	35.7 ± 2.1	31.3 ± 3.2	181.7 ± 19.6	196.3 ± 0.6	265.7 ± 4.2	306.3 ± 0.6	22.0 ± 3.5	17.0 ± 0.0	6.3 ± 2.5	4.0 ± 1.7
316	32.0 ± 1.7	35.0 ± 5.6	186.3 ± 2.1	189.3 ± 6.7	272.0 ± 9.0	294.7 ± 5.7	23.7 ± 1.2	19.0 ± 2.0	5.0 ± 1.7	4.7 ± 1.5
600	35.0 ± 1.7	35.3 ± 3.1	186.7 ± 4.9	187.3 ± 7.5	270.7 ± 30.2	251.3 ± 2.1	23.3 ± 8.1	19.7 ± 1.5	6.3 ± 2.1	5.0 ± 2.6
Positive control[a,b]	186.3 ± 6.0	172.0 ± 36.3	883.7 ± 3.5	797.0 ± 81.3	1001.3 ± 4.7	990.3 ± 44.2	173.3 ± 1.5	179.0 ± 3.0	76.7 ± 4.9	73.3 ± 1.5

Abbreviations: BaP, benozo[a]pyrene; 2-AA, 2-aminoanthracene; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; NaN_3, sodium azide.
Values are means (n=3) ± standards deviations.
[a]Positive controls without S9: NaN_3 for TA1535 and TA100, 2-NF for TA98, 9-AA for TA1537, mitomycin C for TA102.
[b]Positive controls with S9: BaP for TA98, TA102 and TA1537, 2-AA for TA100 and TA1535.
[c]The HMO mixture containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein.

ii. *In vitro* Micronucleus Test

To evaluate the clastsrogenicity and/or aneugenicity of an HMO mixture containing 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, Parschat et al. (2020) performed an OECD-compliant *in vitro* micronucleus test using human peripheral blood lymphocytes. Peripheral blood lymphocytes were obtained by venipuncture from young, healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation and exposed to 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) for 4 or 24 hrs in the presence and absence of metabolic activation. Purified water was the negative control and the positive controls were mitomycin C (at 0.2 μg/mL), colchicine (at 0.02 μg/mL), and cyclophosphamide (at 20 μg/mL) with and/or without metabolic activation. At least 500 cells per replicate cell culture were scored and classified as mononucleates, binucleates, or multinucleates to estimate the proliferation index as a measure of toxicity. The evaluation of cytotoxicity was based on the Cytokinesis-Block Proliferation Index (CBPI) or the Replicative Index (RI). The CBPI indicates the average number of nuclei per cell during the period of exposure to CytoB and is used to calculate cell proliferation. The RI indicates the relative number of cell cycles in treated cultures compared to control cultures and can be used to calculate the percentage of cytostasis. Micronucleus frequencies were analyzed in at least 2000 binucleate cells per concentration (*i.e.*, ≥ 1000 binucleate cells per culture; two cultures per concentration). The ability of the HMO mix to induce micronuclei was considered to be positive if there was a statistically significant and/or dose related increase compared to the negative control or if any of the results were outside the distribution of the historical negative control data (Poisson-based 95% control limits).

Mitomycin C and cyclophosphamide induced significant chromosomal damage whereas colchicine induced significant ($p \leq 0.05$) damage to the cell division apparatus, both validating the tests. In contrast, no chromosomal damage was observed with the HMO mixture at any concentration or under any condition tested (Table 11). Thus, the HMO mixture was not genotoxic under the tested conditions at concentrations up to 60 mg/mL (14.2 mg/mL LNT).

Table 11. *In vitro* Micronucleus Test in Human Peripheral Blood Lymphocytes Exposed to an HMO Mixture Containing 23.7% Lacto-*N*-Tetraose[b]

HMO Mixture (mg/mL)	CBPI	RI (%)	Number of binucleate cells scored	Number of micronucleated cells per 1000 binucleate cells
4-h treatment −S9				
Negative control (water)	1.96	100	2000	4.0
7.5	1.83	87	2000	5.0
15	1.84	88	2000	4.5
30	1.99	103	2000	8.5
60	1.85	88	2000	6.0
Mitomycin C (0.2 µg/mL)	1.77	80	2000	44.5[a]
24-h treatment −S9				
Negative control (water)	1.58	100	2000	2.5
7.5	1.48	81	2000	3.5
15	1.56	95	2000	4.5
30	1.57	98	2000	2.5
60	1.31	53	2000	5.0
Colchicine (0.02 µg/mL)	1.57	96	2000	17.0[a]
4-h treatment +S9				
Negative control (water)	1.62	100	2000	4.0
7.5	1.59	97	2000	3.5
15	1.61	99	2000	2.0
30	1.57	93	2000	2.0
60	1.57	93	2000	2.0
Cyclophosphamide (20 µg/mL)	1.40	65	2000	26.5[a]

Values are means (n = 2)
CBPI = Cytokinesis block proliferation index; RI = Replicative Index.
[a]Significantly different from negative control ($p \leq 0.05$)
[b]The HMO mixture also contains 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein.

2. Subchronic Toxicology Studies

a. Studies on Lacto-N-Tetraose as a Single Ingredient

As summarized on pg 34 of GRN 833, a 14-day dose-range finding oral toxicity study was conducted in neonatal rats with powder containing 77% LNT manufactured by Glycom A/S. The study was conducted to inform the design of a subsequent 90-day subchronic oral toxicity study. Neonatal rats were an administered either a vehicle or 3,250 or 4,000 mg LNT/kg body weight/day by gavage (n=8/sex/group). All animals were observed daily for changes in clinical condition. Body weights were recorded daily until the end of the dosing period, when animals were subjected to a gross macroscopic necropsy.

One male in the 4000 mg/kg bw/day group was found dead on Day 14 of dosing. Because there were no changes in clinical condition, no macroscopic abnormalities at necropsy and no evidence of dosing trauma, the death was considered incidental and unrelated to the LNT

treatment. There were also no test item-related clinical signs, no biologically relevant differences in body weight between test item-treated groups and controls, and no test item-related macroscopic abnormalities at necropsy in the remaining animals. As a result, 4,000 mg/kg body weight/day was considered the maximum feasible dose, based on viscosity, and was chosen to be the high dose in 90-day study oral toxicity study.

As summarized in on pg. 35 or GRN 833, Phipps et al. (2018) evaluated the subchronic oral toxicity of powder containing 77% LNT, manufactured by Glycom A/S, in an adapted OECD 408-compliant 90-day oral toxicity study. Neonatal Crl:CD(SD) rats were administered either vehicle, 1,000, 2,500, or 4,000 mg/kg body weight/day of LNT by gavage once daily for 90 days, until the day before necropsy (n=15/sex/group). Five male and female mice in each group were left undosed after the 90-day treatment period to assess the reversibility of any observed effects. An additional group (n=10/sex) received FOS via gavage at 4,000 mg/kg body weight/day under the same conditions to allow for direct comparison against the high-dose LNT group and identify any effects related to the fiber-like characteristics of the LNT material (n=10/sex/group). Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of treatment until weaning and twice weekly thereafter. Feed intake was recorded twice weekly from weaning until necropsy. The eyes of vehicle-, FOS- and high-dose LNT-treated animals were examined in Week 13. Blood samples were taken for hematology, blood chemistry and coagulation during Week 13 and at the end of the treatment-free period. Additional blood samples were taken at the end of the treatment period for potential analysis of thyroid stimulating hormone (TSH), T3 and T4 and were stored frozen until the end of the study or until analyzed. Urinalysis was conducted in Week 13 and at the end of the treatment-free period. In Week 11/12, all animals were subjected to a functional observational battery consisting of observations in-hand and in a standard area, in addition to an assessment of grip strength and learning and memory (using the Morris water maze). Pre-weaning reflex development (eye opening, air righting, startle response and pupil closure response), ulna length and sexual maturation (balano-preputial separation and vaginal opening) were also recorded for all animals. All surviving animals (at the end of the treatment and recovery periods) were subjected to a gross macroscopic necropsy, selected organs/tissues were weighed and fixed. At the end of the treatment period, the organs/tissues for early decedents and animals in the vehicle-, high-dose LNT-, and FOS-treated groups were examined microscopically.

There were six deaths (1 male and 1 female in the vehicle-treated group, 1 male and 1 female in the 1,000 mg LNT /kg body weight/day-treated groups, 1 female in the 4,000 mg LNT /kg body weight/day-treated group, and 1 female in the FOS-treated group) over the course of the study. Three of the deaths (one female from the vehicle-, 1000 mg/kg bw/day-, and FOS-treated groups) were considered to be the result of dosing trauma. For the remaining three animals,

there was no specific cause of death (i.e, the male from the vehicle-treated group was euthanized on Day 3 due to excessive weight loss, but no macro or microscopic findings; the male in the 1000 mg/kg bw/day group died during Morris maze testing on Day 79 and the macroscopic findings included red fluid around the trachea, incomplete collapse of the lungs, dark areas on the ventricles of the lungs, diaphragmatic herniation, a mass on the medial lobe of the liver, and pale glandular mucosa of the stomach; the female from the 4000 mg/kg bw/day-treated group was found on day 39 and a majority of the tissues were cannibalized). However, because the macro- and microscopic findings in these three animals were not LNT-related, the deaths were deemed to be incidental and not LNT-related.

There were also no LNT-related clinical signs or ocular findings and although there was a statistically significant 8% reduction in mean feed intake in the males treated with 4000 mg/kg bw/d compared to the vehicle-treated males, the mean overall intake value (23 g/animal/day) was the same for the males in the FOS-treated group over the same period and all LNT-treated animals gained similar amounts of weight of the course of the study. Thus, the 8% reduction in mean feed intake was deemed to be not LNT-related. Additionally, no biologically relevant differences in feed consumption were observed during the recovery period.

A significant increase in the body weights of the females in 2500- and 4000 mg/kg bw/day-treated group was observed on the day of vaginal opening; however, the difference was considered to be unrelated to LNT administration due to the lack of a dose response and lack of difference in the age at which vagina opening occurred in the different groups. LNT administration also had no effect on pre-weaning development, ulna length and growth, or neurobehavior.

A variety of significant differences in hematological and blood chemistry values were also observed in the LNT-treated animals compared to controls (i.e. changes in red blood cell (RBC)-, white blood cell (WBC)-related parameters and platelets) and blood chemistry parameters (i.e. changes in transaminases, urea, creatinine, cholesterol and electrolytes levels), however, the differences were not dose-dependent, within the historical control range for the laboratory, only occurred in one gender, and/or occurred at the end of the recovery period. Thus, Phipps et al. (2018) considered the effects to be not LNT-related. Similarly, significant differences in selected urinalysis parameters were observed such as in urine volume and specific gravity; however, they were deemed to be normal biological variation because all individual values for these parameters were within the historical control range for the age and strain of rat at the testing facility.

Additionally, although significant changes in organ weights (i.e., kidney, liver, ovary and spleens) and body weight-relative organ weights (testes) were observed in the LNT-treated

groups compared to the vehicle-treated group, the increases were either not dose-dependent or evident immediately after dosing, and not associated with non-incidental histopathological findings.

Based on these results, Phipps et al. (2018) concluded that the NOAEL for LNT was at least 4000 mg/kg bw/day.

b. Toxicity Studies on Jennewein's LNT as Part of an HMO Mixture

i. Seven-day Dietary Toxicity Study

In a seven-day pilot feeding toxicity study, female CD/Crl:CD rats (Charles River Laboratories, Sulzfeld, Germany) received either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Jennewein (n=5/group) (Parschat et al., 2020). All animals were individually housed. The HMO mixture contained 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, all of which were manufactured by fermentation. Thus, the overall dietary exposure to LNT was 2.37% of the diet. Both diets were provided *ad libitum.* Animals were observed daily for viability, behavioral changes, and reactions to treatment or illness. Cage-side observations included skin and fur, eyes, mucous membranes, respiratory and circulatory systems, somatomotor activity, behavior patterns, and feces output and consistency. Body weight was recorded at the time of group allocation, on the 1st day of treatment and daily thereafter at the same time each day. Feed consumption was recorded daily and feed intake per rat (g/rat/day) was calculated subtracting the total amount of feed left from the total amount of feed given and dividing the difference by the number of days and body weight of the rat. Drinking water consumption was monitored daily by visual inspection. Intake of the test article was calculated on a daily and weekly basis throughout the experimental period based on the concentration in the diet, individual feed intake and body weight of each rat. No mortalities occurred during the study. No HMO-related differences in behavior, appearance and consistency of the feces, body weight, body weight gain, or feed consumption were observed. Thus, the dose of 10% HMO mixture in diet (23.7 % LNT by dry weight, providing LNT as 2.37% of total diet) was chosen for the subsequent 13-week dietary toxicity study in rats.

ii. Thirteen Week Dietary Toxicity Study

Parschat et al. (2020) fed either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Jennewein to rats for 90 days (n=10/sex/group) in an OECD 408-compliant 90-day dietary toxicity study. The HMO mixture contained 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, all of which were manufactured by fermentation. Thus, the overall dietary exposure to LNT was 2.37% of diet. Both diets were provided *ad libitum.* All animals were individually housed, and observed daily for clinical signs of toxicity and twice daily for mortality. Cage-side observations included changes in the skin, fur, eyes and mucous membranes, the occurrence of secretions or excretions, autonomic activity (e.g. lacrimation, pilo-erection, pupil size, and unusual respiratory patterns), gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviors (e.g. self- mutilation, walking backwards). Clinical observations were made once before the first exposure and weekly thereafter. Body weight was recorded at the start of the adaptation period, at the time of group allocation, on the day treatment commenced, and weekly thereafter at the same time each day. Feed consumption was recorded daily, and feed intake per rat (g/rat/week) and relative feed consumption (g/kg bw/day) were calculated. Drinking water consumption was monitored daily by visual inspection. Neurological screening was conducted in test week 13 before blood sampling to evaluate sensory reactivity to different stimuli (auditory, visual and proprioceptive stimuli), grip strength and to assess locomotor activity. Observational screening included tests covering peripheral, sensory, muscular, central and gastro-intestinal neural components. Functional tests comprised grip strength and locomotor activity. Ophthalmological and auditory examinations were conducted before the study and one week before the end of treatment. Blood and urine samples were taken from overnight fasted animals at the end of test week 13 before necropsy. Blood was collected for hematology, coagulation, and clinical chemistry analyses. Urine was collected for 16 hours and analyzed for volume, pH, specific gravity, protein, glucose, bilirubin, urobilinogen, ketones, hemoglobin, and nitrite. Urine was also analyzed by microscopy for epithelial cells, leucocytes, erythrocytes, organisms, crystalluria, and constituents such as sperm and casts. Color and turbidity of the urine were examined visually.

On test day 90, animals were euthanized, weighed, and inspected macroscopically. The weights of the adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, uterus (including cervix), and prostate and seminal vesicles with coagulating glands as a whole were determined. Histological analysis was carried out on the organs listed above as well as the aorta abdominalis, bone (os femoris with joint), bone marrow (os femoris), eyes with optic nerve, gross lesions observed, large intestine (colon, rectum), small intestine (duodenum, jejunum, and ileum, including Peyer's patches), lungs (with mainstem bronchi and bronchioles), lymph node (cervical and mesenteric), mammary gland, muscle (skeletal, leg), nerve (sciatic), esophagus, pancreas, pituitary, salivary glands (mandibular, parotid, and sublingual), skin (left flank), spinal cord (cervical, midthoracic, and lumbar), stomach, thyroids (including

parathyroids), tissue masses or tumors (including regional lymph nodes), trachea (including larynx), urinary bladder and vagina.

Based on feed consumption, the mean intake of the HMO mixture ranged from 5.01 to 6.88 g/kg bw/day for male rats and 6.26 to 7.91 g/kg bw/day for the female rats. This resulted in a mean intake of LNT of 1.18 to 1.63 g/kg bw/day in males and 1.48 to 1.87 g/kg bw/day in females.

Prior to and over the course of four weeks of the 13-week study, one male animal in the control group (standard diet) gained weight at a slower rate compared to the other control animals. From six days prior to the study to day 29, the male gained weight as a slower rate compared to the remaining rats in the control group. From day 29 to day 90, the body weight remained constant while the remaining control male rats continued to gain weight. This resulted in 12% lower body weight at day 29 and a 27% lower body weight at the end of the study compared to other control males. Although no changes in behavior or external appearance were noted over the course of the study, multiple erosions/ulcerations in the small intestine, thickening of the duodenum wall, white foci in the lungs, enlarged glassy mandibular lymph node, enlarged and thickened mesenteric lymph node, and enlarged spleen were noted at necropsy. Hematology revealed an increased number of leucocytes (9-fold) caused by increased numbers of neutrophilic granulocytes (26-fold), lymphocytes (4-fold), monocytes (19-fold), eosinophilic granulocytes (43-fold), large unstained cells (15-fold) and basophilic granulocytes (24-fold) compared to the mean values for the group. Clinical chemistry revealed increased plasma level of bilirubin (3-fold) and increased enzyme activities of alanine aminotransferase (8-fold), alkaline phosphatase (2-fold), aspartate aminotransferase (12-fold) and lactate dehydrogenase (3-fold). Due to the magnitude of the hematological and clinical chemistry changes, the effects were deemed spontaneous and incidental and the animal was excluded from all analyses.

The HMO mixture had no effect on feed consumption, water consumption, body weight, or body weight gain in either males or females. Except for the one rat that was euthanized moribund and excluded from all analyses, no other mortalities were observed during the study, and no changes in behavior, external appearance, or consistency of feces were recorded in either group. No ophthalmological or auditory changes or effects on body posture, movement, or coordination were observed. Neurological screening revealed no test article-related effects. Although a significant ($p \leq 0.05$) increase in body temperature was reported in female rats in the HMO mix group (38.5 ± 0.3 °C) compared to the control group (38.1 ± 0.4 °C), the decrease was small (approximately 1%), occurred only in females, and was not associated with any other clinical observations. Additionally, male rats in the HMO mix group showed a significant decrease ($p \leq 0.05$) in spontaneous motility (number of movements recorded over a period of 12 min), with a mean value of 96.3 ± 50.3 compared to 167.7 ± 73.9 in the control male rats.

Further inspection of the individual rat data revealed that the decrease was due to two males in the control group having spontaneous motilities higher than the upper boundary of the historical range for the laboratory (224 and 299 movements/12 min vs an upper boundary of 217 movements/12 min; laboratory historical control mean of 77.7 movements/12 min). Thus, the increase in body temperature and decrease in spontaneous mobility were deemed to be incidental and not related to the HMO mixture.

Except for a statistically significant reduction ($p \leq 0.05$) in the absolute number of neutrophilic granulocytes in female rats receiving the HMO mix compared to the control ($0.71 \pm 0.38 \times 10^3$ vs $0.80 \pm 0.2 \times 10^3$ cells/μl), there were no significant differences between the control and HMO mix groups in any of the remaining hematological parameters. There were also no significant differences between the groups in the myeloid/erythroid ratio in the bone marrow.

For the neutrophils, the mean cell counts were generally low relative to the historical control range for the laboratory ($0.4\text{-}12.81 \times 10^3$ cells/μl) in both the control and HMO mix groups. Additionally, although the absolute number in one female receiving the HMO mix fell below the lower boundary of the historical control range (0.33×10^3 cells/μl), all neutrophil counts in the remaining males and females fell within the historical range. Thus, the statistically significant reduction in the absolute number of neutrophilic granulocytes observed in female rats administered HMO mix was deemed to be unrelated to test article administration.

Statistically significant changes were also noted in selected clinical chemistry parameters in male and female rats receiving the HMO mixture compared to the males and females receiving the control diet (Table 12). Specifically, in the HMO-treated males, significant increases in HDL-C were observed, although the levels overall were within the historical range for the laboratory and this species. In the HMO-treated female rats, plasma levels of albumin ($p \leq 0.05$), globulin ($p \leq 0.01$), total protein ($p \leq 0.01$), urea ($p \leq 0.01$), and the plasma albumin/globulin ratio ($p \leq 0.05$) were significantly increased while ALT was significantly decreased ($p \leq 0.05$) compared to the control group. All means for these parameters were within the historical range for the laboratory and the species, and not greater than 15% different from the control group means. Importantly, because the plasma albumin, globulin, protein, urea, and albumin/globulin ratio values were all within the historical range for the laboratory and the species, and small in magnitude ($\leq 15\%$), these changes were deemed unrelated to the HMO mixture.

Table 12. Statistically Significant Differences in Clinical Chemistry Values on Day 92

Sex	Treatment	Alb [g/L]	Glob [g/L]	Alb/Glob	HDL-C [mmol/L]
M	Control (N)	29.8 ± 0.7 (9)	30.9 ± 2.4 (9)	0.98 ± 0.06 (9)	0.66 ± 0.18 (9)
F	Control (N)	34.2 ± 2.3 (10)	34.9 ± 3.4 (10)	0.98 ± 0.06 (10)	0.70 ± 0.12 (10)
M	10% HMO (N)	29.3 ± 0.6 (10)	30.4 ± 1.2 (10)	0.97 ± 0.03 (10)	0.92 ± 0.29 (10) [a,$]
F	10% HMO (N)	32.2 ± 1.1[a,$] (10)	30.9 ± 1.3[b,$] (10)	1.05 ± 0.04[a,$] (10)	0.77 ± 0.18 (10)
Sex	**Treatment**	**TP [g/L]**	**Urea [mmol/L]**	**ALT [U/L]**	
M	Control (N)	60.7 ± 2.9 (9)	4.7 ± 0.6 (9)	39.6 ± 7.7 (9)	
F	Control (N)	69.1 ± 5.5 (10)	5.0 ± 0.4 (10)	40.7 ± 13.3 (10)	
M	10% HMO (N)	59.7 ± 1.6 (10)	5.2 ± 0.7 (10)	35.8 ± 9.0 (10)	
F	10% HMO (N)	63.1 ± 2.0[b,$] (10)	5.8 ± 0.6[b,$] (10)	30.9 ± 8.2[a,$] (10)	

Abbreviations: N, number of animals per sex and group; M, male; F, female; HMO: human milk oligosaccharide mixture containing 16.0% 3-fucosyllactose (dry weight); Alb, albumin; Glob, Globulin; TP, total protein; HDL-C, high density lipoprotein cholesterol; ALT, alanine aminotransferase.
Values are means ± standard deviations.
[a]Significantly different from control ($p \leq 0.05$).
[b]Significantly different from control ($p \leq 0.01$).
[$]Laboratory Historical Control Ranges: Alb (27.2-37.5 g/L); Glob (26.8-37.7 g/L); Alb/Glob (0.72-1.19); TP (54.0-75.0 g/L); Urea (3.73-7.76 mmol/L); ALT (20.0-175.0 U/L); HDL-C (males: 0.42-2.36 mmol/L; females: 0.09-0.48 mmol/L).

Urinalysis on test day 92 revealed no changes in any of the parameters except for a statistically significant decrease ($p \leq 0.05$) in the specific gravity of urine from female rats in the HMO-treated group. This decrease was small (approx. 1%) and within the historical range for the laboratory. Because of these factors, the difference in specific gravity was deemed unrelated to test article administration.

Macroscopic inspection at necropsy did not reveal any test item-related changes in the organs or tissues of any animal, with the exception of one animal from the control group. As stated above, this control male was excluded from all statistical evaluations.

Some statistically significant differences in absolute and relative organ weights were noted between control and the HMO mixture-treated groups (Table 9 and Table 10, respectively). Specifically, the absolute weight of the brains in HMO-treated male rats were lower ($p \leq 0.05$), the absolute weights of the right kidneys were lower in HMO-treated female rats ($p \leq 0.05$), and the relative weights of the left and right kidneys were lower in the HMO-treated female rats ($p \leq 0.05$). There were no significant differences in the absolute and relative weights of the other organs examined. Review of the individual animal data revealed that one female rat in the HMO-treated group had an absolute weight of the right kidney less than the lower boundary of the historical range for the laboratory. The left kidney of the same animal was also small relative to the other rats in the group (0.79 g versus a range of 0.92-1.12 g for the other female rats) and approached the lower boundary of the historical range (0.78-1.40 g).

Together, these results indicated that the kidneys in this individual female were generally smaller than other rats in the HMO-treated group. None of the absolute or relative organ weight changes in the HMO-treated rats were associated with histopathologic changes. Therefore, because the brain and kidney changes were within the historical range for the laboratory, the kidney changes in the HMO group were exaggerated by a single animal with small kidneys, and the changes in the absolute and relative organ weights were not associated with adverse clinical chemistry effects or histopathologic changes, the significant differences in the absolute and relative organ weights in the HMO-treated group were deemed as normal biological variation.

Table 13. Significant Differences in Mean Brain and Kidney Weights

Sex	Treatment	Brain [g]	Kidney (r) [g]
M	Control (N)	2.2 ± 0.1 (9)	1.9 ± 0.1 (9)
F	Control (N)	1.9 ± 0.1 (10)	1.1 ± 0.1 (10)
M	10% HMO (N)	2.1 ± 0.1[a,$] (10)	1.6 ± 0.1 (10)
F	10% HMO (N)	2.0 ± 0.1 (10)	1.0 ± 0.1[a,$] (10)

Abbreviations: N, number of animals; M, male; F, female; (r), right; HMO: human milk oligosaccharide mixture containing 16.0% 3-fucosyllactose (dry weight).
Values are means ± standard deviations.
[a]Significantly different from control ($p \leq 0.05$).
[$]Laboratory Historical Control Ranges: Brain (1.76-2.35 g); Kidney (r)(0.85–1.48 g).

Table 14. Significant Differences in Mean Relative Kidney Weights

Sex	Treatment	Left	Right
M	Control (N)	3.8 ± 0.3 (9)	3.8 ± 0.2 (9)
F	Control (N)	4.2 ± 0.1 (10)	4.2 ± 0.4 (10)
M	10% HMO (N)	3.5 ± 0.3 (10)	3.6 ± 0.3 (10)
F	10% HMO (N)	3.8 ± 0.4[a,$] (10)	3.8 ± 0.4[a,$] (10)

Abbreviations: N, number of animals; M, male; F, female; HMO: human milk oligosaccharide mixture containing 16.0% 3-fucosyllactose (dry weight).
Values are means ± standard deviations.
[a]Significantly different from control ($p \leq 0.05$).
[$]Laboratory Historical Control Ranges: Kidney (l) (2.94-5.03 g); Kidney (r) (2.95-5.32 g).

An uncertain test-item related histopathologic finding was present in the livers of males that had *ad libitum* access to a diet containing the HMO mix. Within the livers of 7 out of 10 males in this group, minimal to slight hepatocellular (ORO-positive) lipid content was noted in the periportal areas mainly, while only 3 males in the standard control group showed the presence of minimal ORO positive fat vacuoles. This marginal change is believed to possibly reflect a change in energy homeostasis known to be associated with an increase in sugar intake in rats (Burgeiro et al., 2017). Because females did not show such an increase and the increase in

lipid content in the males was not associated with any other liver pathology, the finding was considered to be not adverse or of toxicologic relevance. No other differences in histopathological observations were observed between the HMO mixture and control groups.

Overall, no signs of toxicity were observed following the administration of an HMO mixture (containing 23.7% LNT by dry weight) at 10% of diet for 13 weeks. Based on feed intake data, the 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, resulting in a mean intake of LNT of 1.34 g/kg bw/day in males and 1.65 g/kg bw/day in females.

D. TOLERANCE STUDY IN NEONATAL PIGLETS

1. Introduction

To understand the safety and tolerance of a mixture containing Jennewein-manufactured HMOs (2'-FL, 3-FL, LNT, 3'-SL and 6'-SL; oligosaccharide blend) in a species other than the rat, a 21 day-neonatal piglet study was conducted. One animal died during the study due to a non-HMO-related bacterial infection. The mixture was well tolerated and did not produce any adverse effects on growth, development, hematology, clinical chemistry, organ weights, gross pathology or histopathology at levels up to 8 g/L. Although this study has not been published, the results corroborate the lack of adverse effects seen in the subchronic chronic oral toxicity study conducted by Phipps et al. (2018) with LNT and the subchronic rat dietary toxicity study conducted by Parschat et al. (2020) using a mixture of HMOs that contained LNT.

2. Materials and Methods

The study was conducted in accordance with the United States (US) Food and Drug Administration (FDA) Good Laboratory Practice (GLP) Regulations, 21 Code of Federal Regulations (CFR) Part 58. The exceptions from the above regulations were: 1) characterization of the bulk test article was performed by the Sponsor or Sponsor subcontractor at a laboratory that follows FDA Good Manufacturing Practice (GMP) regulations and was not considered to have had an adverse impact on the quality or integrity of the study; 2) dose formulation analyses performed by the Sponsor were not conducted according to GLP regulations. The dose formulations analyses were performed following standard operating procedures using analytical methods developed by the Sponsor for this compound; therefore, these evaluations were not considered to have had an adverse impact on the quality or integrity of the study.

The objective of this study was to evaluate the potential effects of the test article, Oligosaccharide Blend, when administered in milk replacer formula to preweaning farm piglets for 3 weeks right after birth (Lactation Day [LD] 2) on growth and development with emphasis

on the gastrointestinal tract. The design of this study was based on the FDA Guidance for Industry: Nonclinical Safety Evaluation of Pediatric Drug Products, the European Medicines Agency (EMEA) Guideline, and was conducted in accordance with all applicable sections of the Final Rules of the Animal Welfare Act regulations (Code of Federal Regulations, Title 9), the Public Health Service Policy on Humane Care and Use of Laboratory Animals from the Office of Laboratory Animal Welfare, and the Guide for the Care and Use of Laboratory Animals from the National Research Council. The pig was selected specifically for use in this study because of the similarity of the digestive systems between swine and humans. The total number of animals to be used in this study is considered to be the minimum required to properly characterize the effects of the test article and the study protocol has been designed such that it does not require an unnecessary number of animals to accomplish its objectives.

Test system: Thirty-six experimentally naïve Domestic Yorkshire Crossbred Swine (farm pig) (18/sex) were received from Bailey Terra Nova, Schoolcraft, Michigan. The animals assigned to study weighed between 1.5 and 2.5 kg at receipt. The day all piglets of a litter were delivered was designated as LD 0. The piglets were transferred to the Testing Facility on LD 2 which was designated as Study Day 1. Prior to receipt, the piglets were given injections of an iron supplement and a broad-spectrum antibiotic injection (EXCEDE® for Swine (ceftiofur crystalline free acid, or equivalent). Animals were transported in a temperature-controlled vehicle from the supplier to the Testing Facility. An additional iron supplement injection was given to all animals approximately 1 week following the initial injection by the supplier. Additional antibiotic injections (LA200 (oxytetracycline injectable solution)) were given via intramuscular injection weekly during the study at a dose volume of 5 mg/kg. All animals were assigned to groups upon receipt; no formal random was conducted.

Control and Test Articles: The control used in the study was ProNurse® (Land O'Lakes Purina Feed, LLC) mixed with deionized water. The test article was an "oligosaccharide blend" containing 49.1 % 2'-FL, 10.4 % 3-FL, 19.9% LNT, 3.5 % 3'-SL and 4.17 % 6'-SL, resulting in a total oligosaccharide content of 87%. Formulations of the test article were prepared by mixing the appropriate amount of ProNurse® with the appropriate amount of test article to achieve nominal concentrations of 5.75 and 8 g/L, which resulted in 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 5.75 g/L formulation and 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 8 g/L formulation. Both formulations were prepared daily and stored refrigerated at 2°C to 8°C. Dosing formulations prepared for the study were evaluated for homogeneity and concentration by collecting samples from the top, bottom, and middle of the formulations using a syringe and 150 mm (Day 1) or 24 inch (Day 20) sampling tube, while stirring, and quantifying the total amount of HMOs.

Administration of Test Materials: Starting on the day of receipt (Day 1), the control and test articles were offered orally via a feeding container 6 times per day (3 hours ±15 minutes between each dose) at a dose volume of 500 ml/kg/day for up to 21 days. The control group received the control article in the same manner as the treated groups.

The study design was as follows (Table 15):

Table 15. Experimental Design

Group No.	Dose Concentration (g/L)	Dose Volume (mL/kg/day)	Number of Animals	
			Males	Females
1[a]	0[a]	500	6	6
2[b]	5.75	500	6	6
3[b]	8.0	500	6	6

[a] Group 1 received ProNurse® only.
[b] Groups 2 and 3 received ProNurse® with Oligosaccharide Blend

Clinical Observations: All animals were observed for morbidity, mortality, injury, and the availability of feed and water twice daily, once in the morning and once in the afternoon. The animals were removed from the cage, and a detailed clinical examination (skin, fur, eyes, ears, nose, oral cavity, thorax, abdomen, external genitalia, limbs and feet, respiratory and circulatory effects, autonomic effects such as salivation, and nervous system effects including tremors, convulsions, reactivity to handling, and unusual behavior) of each animal was performed twice weekly, prior to the first feeding during the study.

Body Weights: Body weights for all animals were measured and recorded daily prior to the first daily feeding throughout the study.

Feed Consumption: Feed consumption was quantitatively measured daily throughout the dosing period; feed efficiency and compound consumption were calculated for each day that feed consumption was measured.

Clinical Pathology: Hematology, coagulation, clinical chemistry and urinalysis sample collection were performed as detailed in Table 16.

Table 16. Clinical Pathology Sample Collection Plan

Group No.[a]	Time Point(s)	Hematology	Coagulation	Chemistry	Urinalysis
1	Day 7 and Day 21	X	X	X	X[b]
2	Day 7 and Day 21	X	X	X	X[b]
3	Day 7 and Day 21	X	X	X	X[b]
Unscheduled Euthanasia	On occasion samples were collected from animals with an unscheduled euthanasia.				
Target Volume (mL)[c]:	NA	1 mL	1.8 mL	1.8 mL	All available
Method:	Urine samples were collected via cystocentesis at necropsy.				
Collection Site:	Anterior vena cava through the thoracic inlet				
Fasting Required:	Water was not available to the animals as the dosing formulations contain sufficient water for the piglets. Animals were not fasted prior to collection.				
Anticoagulant:	NA	K_2EDTA	Sodium Citrate	Serum Gel Separator	NA

X = Sample was collected; NA = Not applicable
[a]Animals were bled at each time point with the exception of collections impacted by unscheduled deaths.
[b]Day 22 at necropsy only.
[c]Additional blood samples were obtained due to sample quality or volume as permissible. Suitable methods were used for unscheduled collections and/or redraws.

Hematology: The following parameters were measured: leukocytes, erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelets, absolute reticulocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, other cells, and red blood cell distribution width (RDW).

Coagulation values: The following parameters were measured: activated partial thromboplastin time (APTT), prothrombin time, and fibrinogen.

Clinical Chemistry: The following parameters were measured: sodium, potassium, chloride, calcium, phosphorous, total bilirubin, gamma glutamyltransferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glutamate dehydrogenase (GLDH), sorbitol dehydrogenase (SDH), low density lipoprotein (LDL), urea nitrogen, creatinine, total protein, albumin, globulin, albumin/globulin, triglyceride, cholesterol, and glucose.

Urinalysis: The following parameters were measured: volume, specific gravity, and pH.

Gross examination: Animals surviving until scheduled euthanasia were euthanized by an intravenous euthanasia solution administration under sedation followed by a Testing Facility SOP approved method to ensure death. When possible, the animals were euthanized rotating across dose groups such that similar numbers of animals from each group, including controls were necropsied throughout the day. If an animal was in overt pain/distress or appeared moribund and was beyond the point where recovery appears reasonable, the animal was euthanized for humane reasons in accordance with the American Veterinary Medical Association

(AVMA) Guidelines on Euthanasia and with the procedures outlined in the protocol. All animals were subjected to a necropsy examination, which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. The animals were examined thoroughly for external abnormalities including palpable masses.

Organ weights: Body weights and organ weights (brain, thyroid gland, heart, kidney, cecum, colon, rectum, liver, small intestine, spleen, and thymus) were recorded for surviving main study animals at the scheduled necropsy and appropriate organ weight ratios were calculated (relative to body and brain weights). Paired organs were weighed together. The liver was weighed with the gallbladder. The large intestine was excised, cut into its applicable sections, gently rinsed with sterile phosphate buffered saline (PBS), then weighed without contents. The small intestine was excised, cut into 4 equal sections, gently rinsed with sterile PBS, then weighed without contents.

Histology: The aorta, sternum, brain, epididymis, esophagus, eye, gallbladder, adrenal gland, lacrimal gland, Harderian gland, mammary gland, parathyroid gland, pituitary gland, prostate gland, submandibular salivary gland, seminal vesicle, thyroid gland, gut associated lymphoid tissue, heart, kidney, cecum, colon, rectum, larynx, liver, lung, mandibular lymph node, mesenteric lymph node, skeletal muscle, optic nerve, sciatic nerve, ovary, pancreas, skin, small intestine, spinal cord, spleen, stomach, testis, thymus, tongue, trachea, urinary bladder, uterus/cervix, and vagina were collected from all animals and preserved in 10% neutral buffered formalin. The eyes (including the optic nerve) were preserved in Davidson's fixative. The testes and epididymides were preserved in modified Davidson's fixative. Protocol designated tissues were embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin. Histopathological evaluation was performed by a board certified veterinary pathologist. A 5-grade scoring system was used for severity scores and included; minimal, mild, moderate, marked, and severe for gradable findings.

3. Results

Dose Formulation Analyses: Homogeneity and concentration analyses results of the 5.75 and 8.0 g/L formulations prepared on Day 1 and Day 20 ranged from 93.3% to 94.1%, respectively, of the targeted dose levels and confirmed that formulations were homogenous and animals received the appropriate concentrations (Table 17).

Table 17. Analysis of Total Oligosaccharide Content in Dosing Formulations – Days 1 and 20		
Dose Level (g/L)	**Average Calculated Concentration (g/L)[a]**	**Average % Recovery[a]**
0	0.07-0.15	NA
5.75	5.37 – 5.41	93.4 – 94.1
8.0	7.46 – 7.51	93.3 – 93.9

[a]Results are the mean values from two control samples and six samples at each Oligosaccharide Blend dose level from Day 1 and Day 20.
BLQ – below the limit
NA – not applicable

Clinical Observations: No test article-related clinical findings were observed at any of the Oligosaccharide Blend dose levels evaluated. The few clinical findings observed in the treated groups were either similar to those observed in concurrent controls and/or seen infrequently and/or considered common in animals of this species, strain, and age and unrelated to treatment (Table 18). Discolored yellow/watery feces were noted in piglets from all groups and a systemic antibiotic (LA 200 (oxytetracycline injectable solution)) was administered for a period of 3 days during the study to piglets exhibiting a fecal score of 6 (no form, watery texture, and watery composition). A total of 5/12 (4 males and 1 female), 4/12 (2 male and 2 female) and 5/11 (3 male and 2 female) piglets were treated in the control, 5.7 g/L and 8.0 g/L groups, respectively (Table 19).

Table 18. Summary of Detailed Clinical Observations						
Observation Type: All Types	**Males**			**Females**		
From Day 3 (Start Date) to 21 (Start Date)	**0 g/L**	**5.75 g/L**	**8.0 g/L**	**0 g/L**	**5.75 g/L**	**8.0 g/L**
Total Number of Animals	6	6	6	6	6	6
EXCRETION						
Feces discolored, Yellow						
Number of Times Recorded	3	5	2	0	2	1
Number of Animals Affected	2	2	2	-	2	1
Feces soft						
Number of Times Recorded	0	2	1	0	0	0
Number of Animals Affected	-	2	1	-	-	-
Feces watery						
Number of Times Recorded	0	4	1	0	1	1
Number of Animals Affected	-	2	1	-	1	1
EXTERNAL APPEARANCE						
Discharge, Red						
Number of Times Recorded	0	0	0	1	3	1
Number of Animals Affected	-	-	-	1	1	1
Material around eyes, Black						
Number of Times Recorded	4	2	0	2	0	0
Number of Animals Affected	2	1	-	1	-	-
Swelling						
Number of Times Recorded	0	1	2	1	1	0
Number of Animals Affected	-	1	1	1	1	-
Thin						
Number of Times Recorded	1	1	2	0	0	0
Number of Animals Affected	1	1	1	-	-	-
EYE/OCULAR						
Eyelid part/completely closed						
Number of Times Recorded	0	0	3	0	0	0
Number of Animals Affected	-	-	2	-	-	-
PELAGE/SKIN						

Table 18. Summary of Detailed Clinical Observations						
Observation Type: All Types	**Males**			**Females**		
From Day 3 (Start Date) to 21 (Start Date)	**0 g/L**	**5.75 g/L**	**8.0 g/L**	**0 g/L**	**5.75 g/L**	**8.0 g/L**
Abrasion(s)						
Number of Times Recorded	4	1	0	2	14	4
Number of Animals Affected	2	1	-	1	4	1
Scabbed area						
Number of Times Recorded	13	13	3	11	37	5
Number of Animals Affected	4	3	2	4	4	3
Skin discolored, Red						
Number of Times Recorded	2	2	6	3	6	3
Number of Animals Affected	2	2	2	2	2	2
EXCRETION						
Emesis, White						
Number of Times Recorded	2	0	0	0	0	0
Number of Animals Affected	2	-	-	-	-	-
Emesis, Yellow						
Number of Times Recorded	1	0	0	0	0	0
Number of Animals Affected	1	-	-	-	-	-
Feces discolored, Orange						
Number of Times Recorded	0	0	1	0	0	0
Number of Animals Affected	-	-	1	-	-	-
Vomitus, Yellow						
Number of Times Recorded	0	0	0	1	0	0
Number of Animals Affected	-	-	-	1	-	-
PELAGE/SKIN						
Skin warm to touch						
Number of Times Recorded	0	0	0	0	1	0
Number of Animals Affected	-	-	-	-	1	-
Unkempt appearance						
Number of Times Recorded	1	0	1	0	0	0
Number of Animals Affected	1	–	1	-	-	-

Table 19. Piglets Receiving Antibiotic (LA200 (oxytetracycline injectable solution)) During the Study

Dose	Animal #[a]	Sex	Day									
			1	2	3	4	5	6	7	8	9	10
0 g/L	1001	Male								X	X	X
0 g/L	1002	Male								X	X	X
0 g/L	1003	Male							X	X	X	
0 g/L	1004	Male							X	X	X	
0 g/L	1505	Female						X	X	X		
5.75 g/L	2001	Male								X	X	X
5.75 g/L	2002	Male								X	X	X
5.75 g/L	2501	Female								X	X	X
5.75 g/L	2506	Female		X	X	X						
8.0 g/L	3002	Male								X	X	X
8.0 g/L	3003	Male							X	X	X	
8.0 g/L	3004	Male							X	X	X	
8.0 g/L	3502	Female								X	X	X
8.0 g/L	3503	Female							X	X	X	

[a]The animal in the 8 g/L-treated group that euthanized due to a moribund condition on day 7 was not treated with antibiotics.

There were no Oligosaccharide Blend-related deaths. Incidentally, one male at 8.0 g/L (Animal No. 3001) was euthanized in extremis on Day 7 related to poor clinical condition; noteworthy microscopic findings contributory to moribundity/euthanasia of this animal included gastrointestinal mucosal gland dilation/inflammation or subacute inflammation, bacteria (presence of gram negative bacilli) and/or goblet cell hypertrophy/hyperplasia with increased mucus. Additional microscopic findings secondary to/correlative with the poor clinical condition of this male included marked adipose fat atrophy (thin body condition), moderate decreased hematopoietic cellularity in bone marrow, lymphoid depletion (decreased lymphocytes) of various examined lymph nodes, thymus, and spleen. The gastrointestinal microscopic findings in this male were considered incidental based on the lack of similar gastrointestinal changes in any other treated animals. The microscopic findings in this male were consistent with causes of mortality frequently observed in pre-weaned piglets.

Body Weights: Mean body weights in males and females at all dose levels were comparable to concurrent controls and unaffected by treatment with Oligosaccharide Blend (Figure 2; Table 16).

Feed Consumption: Mean feed consumption in males and females at all dose levels evaluated were comparable to concurrent controls and unaffected by treatment with the Oligosaccharide Blend (Figure 3; Table 17). Mean feed efficiency in males and females at all dose levels were comparable to concurrent controls indicating good growth at the concentrations tested with the exception of a statistically lower feed efficiency on Days 18-19 in females at 5.75 g/L (11.00% vs 18.12% in controls). This difference was not dose-dependent and considered unrelated to treatment (Table 18). Calculated compound consumption in both sexes followed the targeted concentrations closely. The high-dose level was about 1.4 times the low-dose level for both sexes over the course of the study (Days 1-21). The calculated compound consumption values for males at 5.75 and 8.0 g/L were 2556.2 and 3576.4 mg/kg/day, respectively. The calculated compound consumption values for females at the same concentrations were 2603.9 and 3659.8 mg/kg/day, respectively.

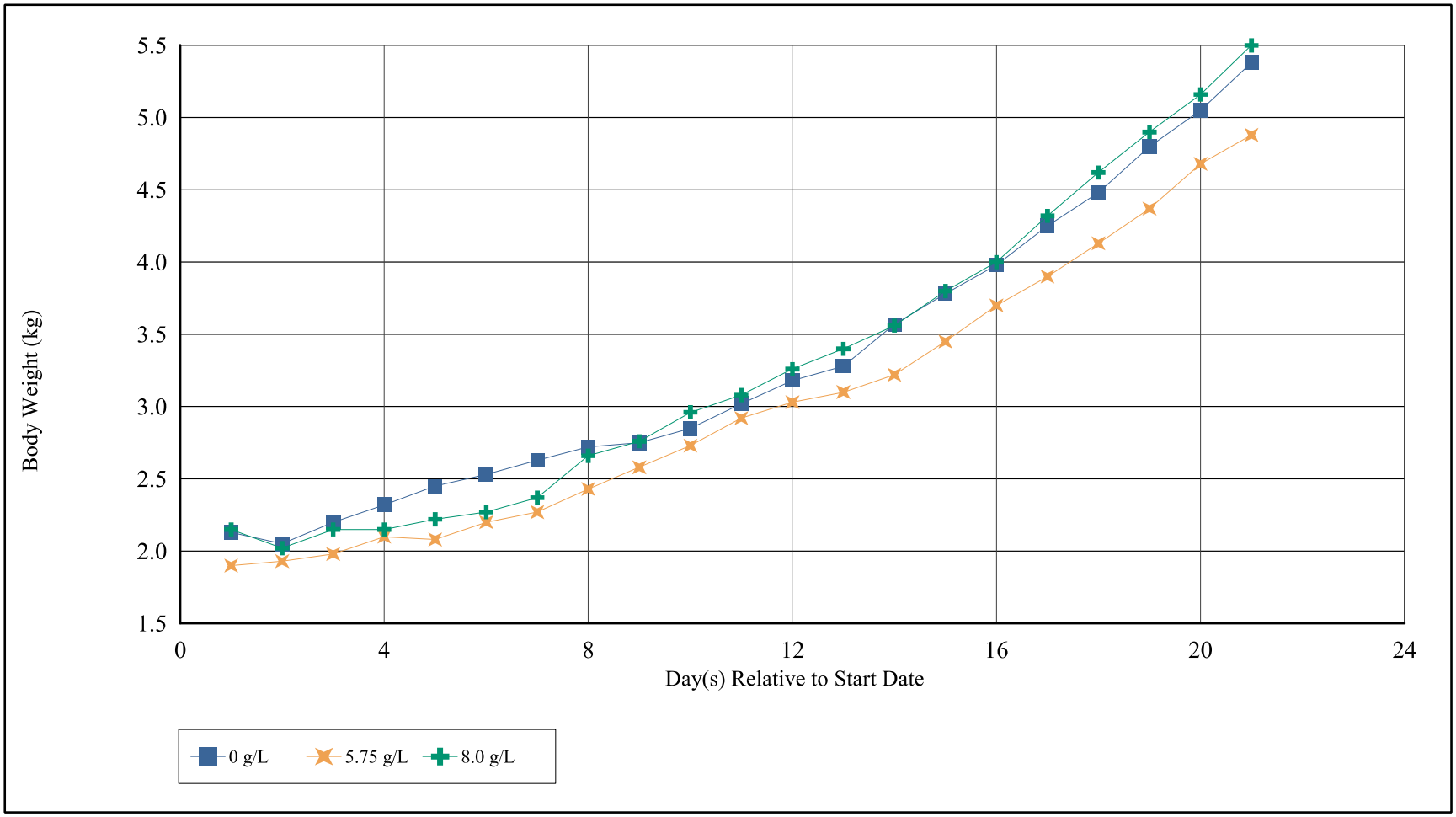


Figure 2a. Mean Body Weight Values (Male)

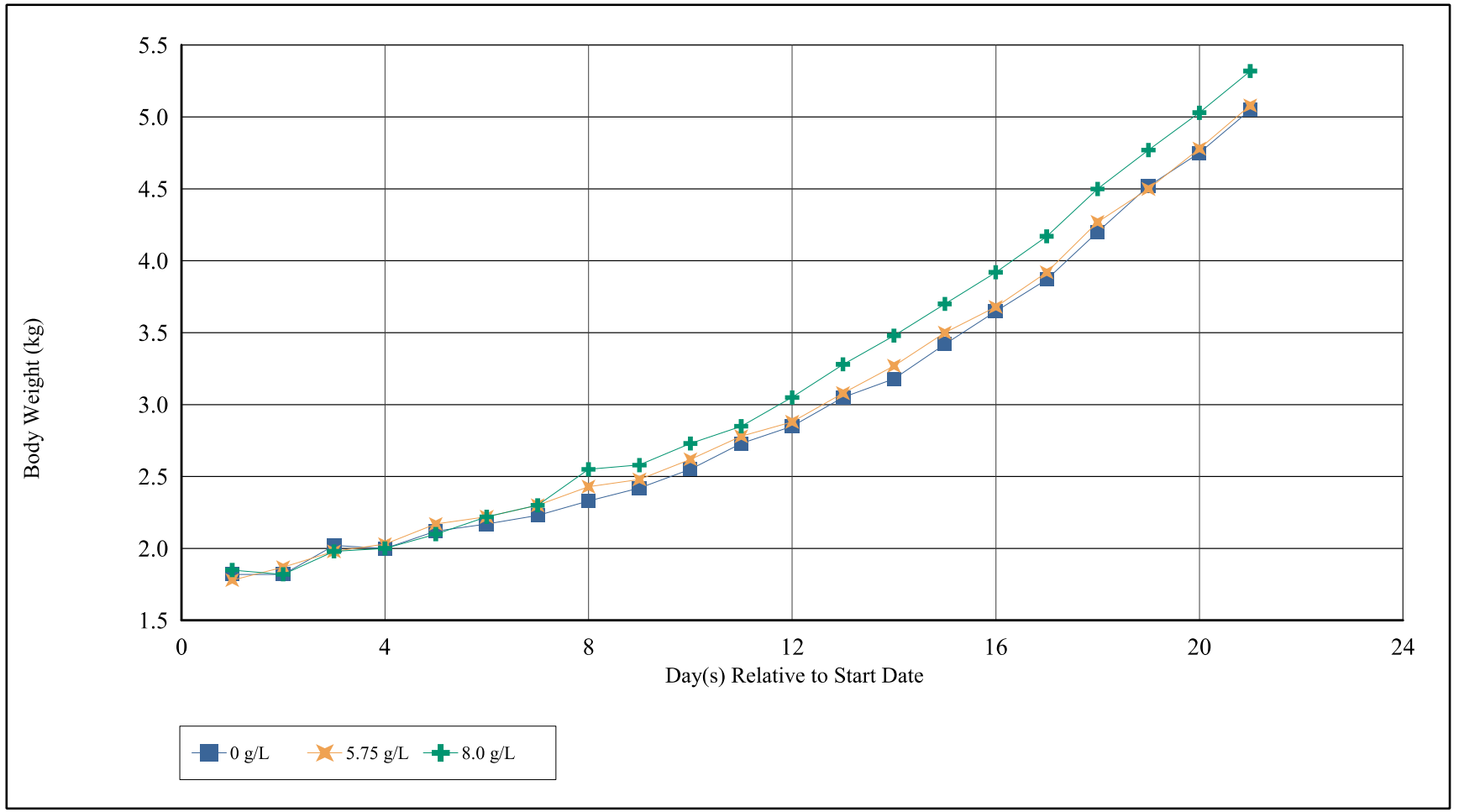


Figure 2b. Mean Body Weight Values (Female)

Table 20. Mean Body Weight Values (kg)						
Day(s) Relative to Start Date	**Males**			**Females**		
	0 g/L	**5.75 g/L**	**8.0 g/L**	**0 g/L**	**5.75 g/L**	**8.0 g/L**
1	2.13 ± 0.234 (6)	1.90 ± 0.063 (6)	2.15 ± 0.226 (6)	1.82 ± 0.204 (6)	1.78 ± 0.160 (6)	1.85 ± 0.207 (6)
2	2.05 ± 0.235 (6)	1.93 ± 0.197 (6)	2.02 ± 0.172 (6)	1.82 ± 0.279 (6)	1.87 ± 0.266 (6)	1.82 ± 0.264 (6)
3	2.20 ± 0.253 (6)	1.98 ± 0.382 (6)	2.15 ± 0.243 (6)	2.02 ± 0.293 (6)	1.98 ± 0.204 (6)	1.98 ± 0.183 (6)
4	2.32 ± 0.293 (6)	2.10 ± 0.473 (6)	2.15 ± 0.207 (6)	2.00 ± 0.341 (6)	2.03 ± 0.288 (6)	2.00 ± 0.237 (6)
5	2.45 ± 0.251 6)	2.08 ± 0.458 (6)	2.22 ± 0.256 (6)	2.12 ± 0.306 (6)	2.17 ± 0.273 (6)	2.10 ± 0.200 (6)
6	2.53 ± 0.344 (6)	2.20 ± 0.469 (6)	2.27 ± 0.372 (6)	2.17 ± 0.308 (6)	2.22 ± 0.271 (6)	2.22 ± 0.232 (6)
7	2.63 ± 0.301 (6)	2.27 ± 0.432 (6)	2.37 ± 0.446 (6)	2.23 ± 0.320 (6)	2.30 ± 0.310 (6)	2.30 ± 0.155 (6)
8	2.72 ± 0.376 (6)	2.43 ± 0.388 (6)	2.66 ± 0.358 (5)	2.33 ± 0.455 (6)	2.43 ± 0.280 (6)	2.55 ± 0.217 (6)
9	2.75 ± 0.451 (6)	2.58 ± 0.407 (6)	2.76 ± 0.391 (5)	2.42 ± 0.479 (6)	2.48 ± 0.319 (6)	2.58 ± 0.232 (6)
10	2.85 ± 0.394 (6)	2.73 ±0.403 (6)	2.96 ± 0.329 (5)	2.55 ± 0.472 (6)	2.62 ± 0.407 (6)	2.73 ± 0.258 (6)
11	3.02 ± 0.417 (6)	2.92 ± 0.479 (6)	3.08 ± 0.349 (5)	2.73 ± 0.532 (6)	2.78 ± 0.454 (6)	2.85 ± 0.308 (6)
12	3.18 ± 0.426 (6)	3.03 ± 0.535 (6)	3.26 ± 0.451 (5)	2.85 ± 0.437 (6)	2.88 ± 0.454 (6)	3.05 ± 0.302 (6)
13	3.28 ± 0.407 (6)	3.10 ± 0.562 (6)	3.40 ± 0.524 (5)	3.05 ± 0.536 (6)	3.08 ± 0.492 (6)	3.28 ± 0.293 (6)
14	3.57 ± 0.450 (6)	3.22 ± 0.519 (6)	3.56 ± 0.650 (5)	3.18 ± 0.527 (6)	3.27 ± 0.463 (6)	3.48 ± 0.343 (6)
15	3.78 ± 0.564 (6)	3.45 ± 0.528 (6)	3.80 ± 0.663 (5)	3.42 ± 0.677 (6)	3.50 ± 0.443 (6)	3.70 ± 0.358 (6)
16	3.98 ± 0.591 (6)	3.70 ± 0.600 (6)	4.00 ± 0.768 (5)	3.65 ± 0.689 (6)	3.68 ± 0.527 (6)	3.92 ± 0.422 (6)
17	4.25 ± 0.635 (6)	3.90 ± 0.678 (6)	4.32 ± 0.756 (5)	3.87 ± 0.726 (6)	3.92 ± 0.640 (6)	4.17 ± 0.476 (6)
18	4.48 ± 0.643 (6)	4.13 ± 0.753 (6)	4.62 ± 0.887 (5)	4.20 ± 0.780 (6)	4.27 ± 0.615 (6)	4.50 ± 0.494 (6)
19	4.80 ± 0.654 (6)	4.37 ± 0.807 (6)	4.90 ± 0.938 (5)	4.52 ± 0.804 (6)	4.50 ± 0.636 (6)	4.77± 0.543 (6)
20	5.05 ± 0.650 (6)	4.68 ± 0.866 (6)	5.16 ± 0.921 (5)	4.75 ± 0.876 (6)	4.78 ± 0.646 (6)	5.03 ± 0.561 (6)
21	5.38 ± 0.717 (6)	4.88 ± 0.900 (6)	5.50 ± 1.068 (5)	5.05 ± 0.935 (6)	5.08 ± 0.685 (6)	5.32 ± 0.571 (6)
ANOVA & Dunnett						

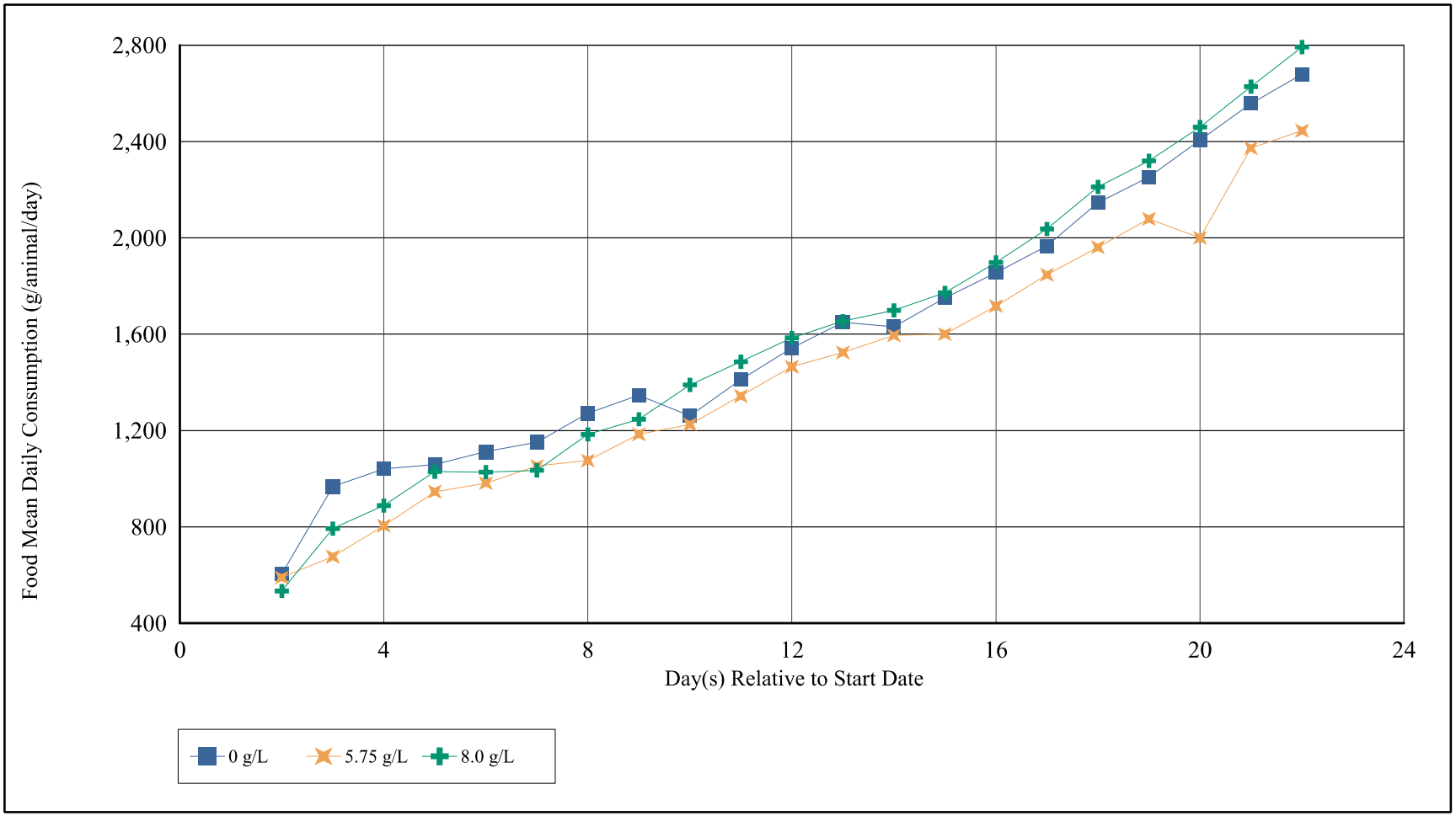


Figure 3a. Mean Feed Consumption Values (Male)

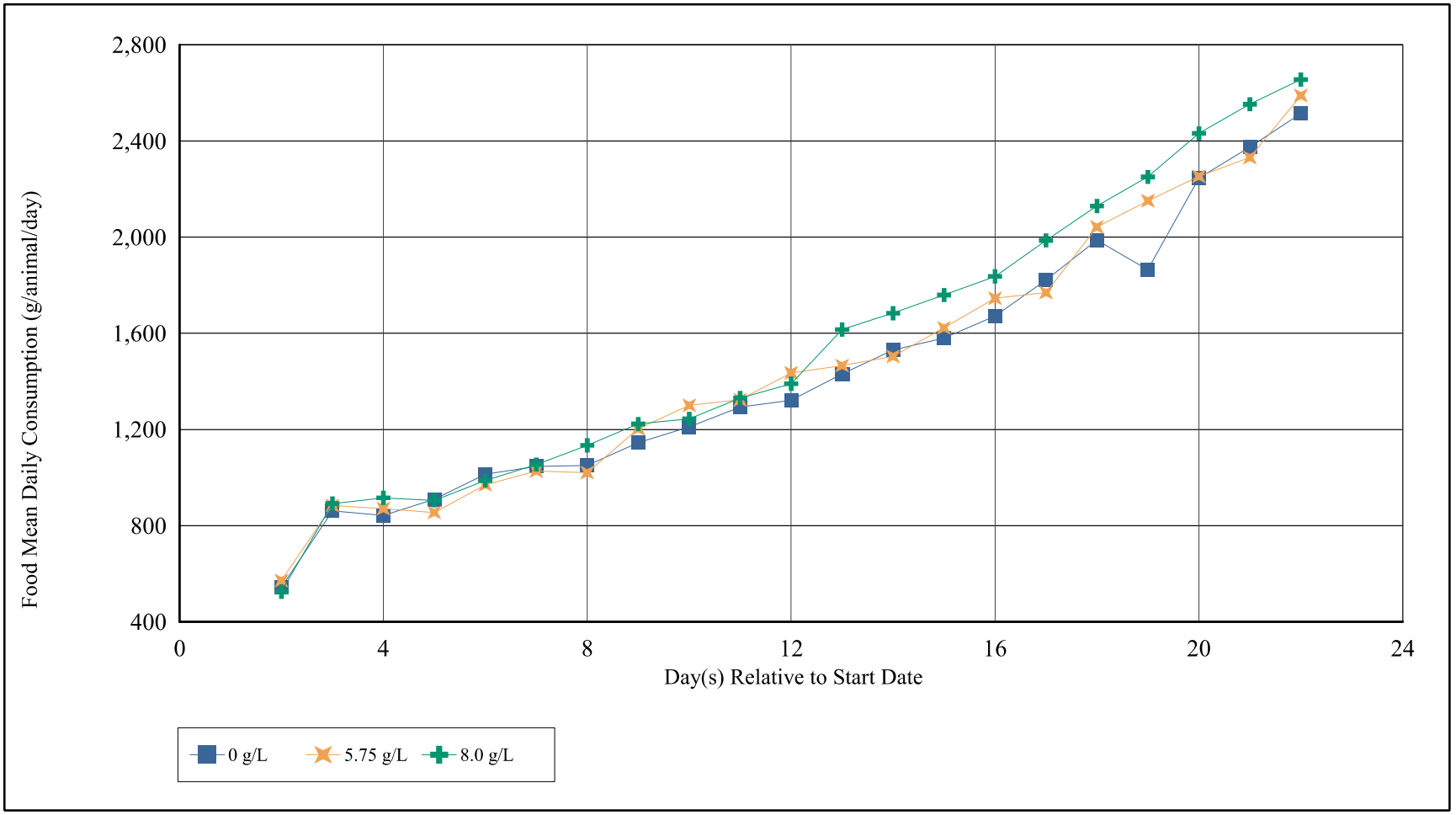


Figure 3b. Mean Feed Consumption Values (Female)

Table 21. Daily Feed Consumption (Mean (g/animal/day) ± St. Dev (n))						
Day(s) Relative to Start Date	**Males**			**Females**		
	0 g/L	**5.75 g/L**	**8.0 g/L**	**0 g/L**	**5.75 g/L**	**8.0 g/L**
1 → 2	603.8 ± 412.90 (5)	589.5 ± 247.63 (6)	533.2 ± 316.30 (6)	543.0 ± 310.13 (5)	571.2 ± 194.95 (6)	525.0 ± 171.01 (6)
2 → 3	967.3 ± 205.08 (6)	676.3 ± 426.52 (6)	792.7 ± 248.01 (6)	861.5 ± 156.45 (6)	884.0 ± 125.41 (6)	892.2 ± 130.20 (6)
3 → 4	1041.0 ± 268.32 (6)	804.7 ± 268.32 (6)	888.3 ± 293.24 (6)	842.0 ± 216.35 (6)	870.8 ± 198.13 (6)	915.5 ± 153.92 (6)
4 → 5	1058.5 ± 186.61 (6)	945.8 ± 354.12 (6)	1029.0 ± 206.03 (6)	909.3 ± 274.15 (6)	854.5 ± 213.76 (6)	905.5 ± 229.61 (6)
5 → 6	1111.7 ± 218.50 (6)	981.3 ± 277.83 (6)	1027.2 ± 287.14 (6)	1013.5 ± 193.93 (6)	969.8 ± 190.85 (6)	987.8 ± 163.28 (6)
6 → 7	1151.8 ± 187.88 (6)	1052.8 ± 271.12 (6)	1034.3 ± 299.42 (6)	1046.5 ± 225.10 (6)	1026.8 ± 153.83 (6)	1054.0 ± 118.23 (6)
7 → 8	1270.8 ± 121.10 (6)	1075.3 ± 286.39 (6)	1183.6 ± 304.03 (5)	1050.5 ± 200.80 (6)	1020.7 ± 207.05 (6)	1133.8 ± 105.68 (6)
8 → 9	1346.3 ± 170.24 (6)	1184.3 ± 238.02 (6)	1246.6 ± 263.92 (5)	1144.5 ± 228.40 (6)	1202.8 ± 134.33 (6)	1223.0 ± 215.09 (6)
9 → 10	1261.5 ± 254.94 (6)	1225.2 ± 214.15 (6)	1389.2 ± 153.68 (5)	1210.5 ± 233.75 (6)	1300.3 ± 165.89 (6)	1244.5 ± 290.58 (6)
10 → 11	1411.3 ± 178.72 (6)	1343.5 ± 229.25 (6)	1485.8 ± 183.65 (5)	1293.3 ± 190.26 (6)	1323.3 ± 195.26 (6)	1331.0 ± 184.68 (6)
11 → 12	1542.3 ± 234.24 (6)	1464.5 ± 211.68 (6)	1584.4 ± 223.44(5)	1321.8 ± 259.14 (6)	1435.0 ± 223.66 (6)	1390.2 ± 253.49 (6)
12 → 13	1649.7 ± 163.52 (6)	1523.8 ± 263.40 (6)	1653.6 ± 258.90 (5)	1430.8 ± 215.56 (6)	1466.0 ± 267.61 (6)	1615.5 ± 265.17 (6)
13 → 14	1631.0 ± 191.46 (6)	1594.5 ± 360.19 (6)	1698.6 ± 253.15 (5)	1530.5 ± 228.34 (6)	1504.0 ± 329.76 (6)	1683.8 ± 171.25 (6)
14 → 15	1750.3 ± 232.71 (6)	1600.2 ± 257.88 (6)	1771.8 ± 322.90 (5)	1580.0 ± 265.36 (6)	1622.5 ± 253.92 (6)	1759.5 ± 156.31 (6)
15 → 16	1855.5 ± 238.22 (6)	1716.5 ± 252.67 (6)	1897.6 ± 318.26 (5)	1672.2 ± 300.05 (6)	1745.8 ± 221.49 (6)	1837.0 ± 152.14 (6)
16 → 17	1966.2 ± 294.68 (6)	1847.0 ± 300.49 (6)	2036.8 ± 469.74 (5)	1821.5 ± 321.11 (6)	1769.5 ± 226.11 (6)	1986.5 ± 239.45 (6)
17 → 18	2145.0 ± 328.66 (6)	1961.2 ± 356.97 (6)	2211.4 ± 375.04 (5)	1987.7 ± 364.71 (6)	2042.0 ± 347.77 (5)	2129.5 ± 233.58 (6)
18 → 19	2251.7 ± 310.40 (6)	2078.8 ± 347.87 (6)	2319.6 ± 414.18 (5)	1864.7 ± 512.55 (6)	2151.5 ± 317.29 (6)	2250.7 ± 188.57 (6)
19 → 20	2406.0 ± 311.99 (6)	2000.2 ± 670.13 (6)	2460.2 ± 476.36 (5)	2246.0 ± 420.50 (6)	2253.2 ± 359.56 (6)	2431.8 ± 294.29 (6)
20 → 21	2557.8 ± 390.17 (6)	2372.5 ± 491.34 (6)	2628.2 ± 492.51 (5)	2374.8 ± 409.67 (6)	2331.2 ± 452.72 (6)	2552.8 ± 457.13 (6)
21 → 22	2679.8 ± 348.53 (6)	2445.0 ± 464.45 (6)	2792.2 ± 487.01 (5)	2514.5 ± 428.24 (6)	2589.2 ± 473.40 (6)	2655.7 ± 313.56 (6)
ANOVA & Dunnett						

Table 22. Feed Efficiency (Mean % ± St. Dev (n))						
Day(s) Relative to Start Date	**Male**			**Female**		
	0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
1 → 2[g]	-25.02 ± 45.510 (5)	-8.03 ± 45.016 (6)	-130.7 ± 279.144 (6)	-3.84 ± 26.295 (5)	10.44 ± 27.735 (6)	-4.24 ± 26.970 (6)
2 → 3[g]	16.52 ± 10.344 (6)	-92.37 ± 249.517 (6)	14.60 ± 32.611 (6)	26.25 ± 25.253 (6)	14.44 ± 14.602 (6)	20.44 ± 17.531 (6)
3 → 4[g1]	10.23 ± 6.490 (6)	11.53 ± 10.676 (6)	0.40 ± 23.839 (6)	-5.88 ± 27.960 (6)	4.98 ± 10.815 (6)	1.52 ± 10.354 (6)
4 → 5[g1]	13.40 ± 9.540 (6)	-0.58 ± 14.073 (6)	6.79 ± 15.784 (6)	15.26 ± 12.873 (6)	16.18 ± 10.676 (6)	12.11 ± 7.292 (6)
5 → 6[g1]	6.48 ± 7.554 (6)	12.61 ± 4.748 (6)	4.14 ± 16.101 (6)	4.97 ± 12.087 (6)	5.50 ± 6.050 (6)	11.71 ± 10.376 (6)
6 → 7[g1]	8.76 ± 10.565 (6)	7.84 ± 13.580 (6)	8.30 ± 7.185 (6)	6.84 ± 5.903 (6)	7.83 ± 6.337 (6)	8.88 ± 14.963 (6)
7 → 8[g1]	6.22 ± 9.032 (6)	17.68 ± 11.168 (6)	14.77 ± 8.311 (5)	8.43 ± 15.712 (6)	13.60 ± 5.816 (6)	22.16 ± 11.106 (6)
8 → 9[g1]	2.31 ± 15.537 (6)	13.54 ± 11.312 (6)	8.15 ± 5.517 (5)	7.26 ± 6.154 (6)	3.95 ± 4.365 (6)	2.64 ± 6.382 (6)
9 → 10[g1]	8.83 ± 6.399 (6)	12.79 ± 9.537 (6)	15.07 ± 11.247 (5)	11.72 ± 8.214 (6)	9.89 ± 6.920 (6)	12.17 ± 3.646 (6)
10 → 11[g]	12.06 ± 10.665 (6)	13.26 ± 6.589 (6)	8.07 ± 2.622 (5)	13.83 ± 8.572 (6)	12.70 ± 12.017 (6)	8.61 ± 4.909 (6)
11 → 12[g]	10.99 ± 7.942 (6)	7.79 ± 4.422 (6)	11.06 ± 9.781 (5)	10.11 ± 8.601 (6)	7.11 ± 3.876 (6)	14.94 ± 6.088 (6)
12 → 13[g]	6.21 ± 5.390 (6)	4.42 ± 6.074 (6)	8.15 ± 7.771 (5)	13.37 ± 6.710 (6)	13.70 ± 3.770 (6)	14.91 ± 6.172 (6)
13 → 14[g]	17.46 ± 8.945 (6)	8.08 ± 7.728 (6)	8.73 ± 6.576 (5)	8.98 ± 5.375 (6)	12.66 ± 7.413 (6)	11.66 ± 4.679 (6)
14 → 15[g]	11.86 ± 4.951 (6)	14.79 ± 3.336 (6)	13.84 ± 3.879 (5)	13.92 ± 10.211 (6)	14.83 ± 7.207 (6)	12.35 ± 2.153 (6)
15 → 16[g]	10.78 ± 6.585 (6)	14.38 ± 3.060 (6)	10.11 ± 4.752 (5)	14.28 ± 3.718 (6)	10.19 ± 5.682 (6)	11.64 ± 3.398 (6)
16 → 17[g]	13.66 ± 3.916 (6)	10.49 ± 3.646 (6)	16.41 ± 4.449 (5)	11.92 ± 3.796 (6)	12.92 ± 9.042 (6)	12.45 ± 3.806 (6)
17 → 18[g]	11.04 ± 7.383 (6)	11.78 ± 5.660 (6)	13.12 ± 5.449 (5)	17.02 ± 6.128 (6)	18.47 ± 8.513 (5)	15.74 ± 2.581 (6)
18 → 19[g]	14.34 ± 6.937 (6)	11.14 ± 3.441 (6)	12.06 ± 2.892 (5)	18.12 ± 5.700 (6)	11.00 ± 5.700 (6)[a]	11.80 ± 3.161 (6)
19 → 20[g]	10.60 ± 3.983 (6)	17.63 ± 8.359 (6)	10.97 ± 3.354 (5)	10.16 ± 2.523 (6)	12.80 ± 2.586 (6)	11.06 ± 4.390 (6)
20 → 21[g]	13.05 ± 4.944 (6)	8.43 ± 5.032 (6)	12.47 ± 4.689 (5)	12.67 ± 3.223 (6)	12.88 ± 5.721 (6)	11.44 ± 4.997 (6)
21 → 22[g]	4.98 ± 7.253 (6)	9.58 ± 3.459 (6)	2.17 ± 8.700 (5)	7.46 ± 2.143 (6)	11.17 ± 2.274 (6)	6.07 ± 7.743 (6)

[g] – Kruskal-Wallis & Dunn
[g1] – ANOVA & Dunnet
a = different from 0 g/L; $p<0.05$

Clinical Pathology:

Hematology: Administration of Oligosaccharide Blend in the diet did not result in test article-related hematological changes (Table 19). Although hematological changes were observed in one male at 8.0 g/L (Animal No. 3001) that was euthanized on Day 7, the changes were incidental and not treatment-related. Other differences in the hematological parameters, were not considered related to Oligosaccharide Blend administration based on their small magnitude, inconsistent direction, absence of a dose response, general overlap of individual values with the range of control values, and/or were of a magnitude of change commonly observed in farm piglets under similar study conditions.

Coagulation: Administration of Oligosaccharide Blend in the diet did not result in test article-related coagulation changes in APTT, prothrombin time or fibrinogen in males or females. All differences in coagulation parameters, regardless of statistical significance, were not considered related to oligosaccharide blend administration based on their small magnitude, inconsistent direction, absence of a dose response, general overlap of individual values with the range of control values, and/or were of a magnitude of change commonly observed in farm piglets under similar study conditions (Table 20).

Clinical chemistry: Administration of Oligosaccharide Blend in the diet did not result in test article-related clinical chemistry changes (Table 21).

On Day 7, individual animals from all treatment groups, including controls, (Animal No. 1001, 1502, 1505, 2001, 2502 and 3002) had lower than expected serum sodium and/or chloride concentrations that were likely secondary to electrolyte loss in the gastrointestinal tract associated with watery feces, which was observed clinically. Changes in serum sodium and chloride concentrations were not considered related to Oligosaccharide Blend administration due to their resolution with continued dosing and occurrence in control animals.

Clinical chemistry changes were also observed on Day 7 in one male at 8.0 g/L (Animal No. 3001) that was euthanized on Day 7 and were considered incidental (Section 3.1).

Other differences in clinical chemistry parameters, regardless of statistical significance, were not considered related to Oligosaccharide Blend administration based on their small magnitude, inconsistent direction, absence of a dose response, general overlap of individual values with the range of control values, resolution with continued dosing, and/or were of a magnitude of change commonly observed in farm piglets under similar study conditions.

Urinalysis: Administration of Oligosaccharide Blend in the diet did not result in test article-related urinalysis changes (Table 22).

Differences in urinalysis parameters were not considered related to Oligosaccharide Blend administration based on their small magnitude, inconsistent direction, absence of a dose-related response, general overlap of individual values with the range of control values, and/or were of a magnitude of change commonly observed in farm piglets under similar study conditions.

Table 23. Hematology (Mean ± St Dev (n))

Parameter	Day	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Leukocytes (10^3 cells/µL)	7 [g]	7.43 ± 1.846 (6)	6.65 ± 1.472 (6)	8.55 ± 4.437 (6)	8.94 ± 2.475 (6)	6.49 ± 1.387 (6)	7.67 ± 1.027 (6)
	21 [g]	10.13 ± 2.114 (6)	8.56 ± 2.488 (6)	8.53 ± 1.010 (5)	9.04 ± 1.907 (6)	8.87 ± 2.578 (6)	10.67 ± 4.078 (6)
Erythrocytes (10^6 cells/µL)	7 [g]	6.083 ± 0.5536 (6)	5.620 ± 0.4502 (6)	5.810 ± 1.0720 (6)	5.818 ± 0.8898 (6)	5.575 ± 0.5443 (6)	5.702 ± 0.6473 (6)
	21 [g]	5.985 ± 0.6187 (6)	5.973 ± 0.4604 (6)	5.572 ± 0.5601 (5)	5.537 ± 0.6020 (6)	5.817 ± 0.4597 (6)	5.847 ± 0.4652 (6)
Hemoglobin (g/dL)	7 [g]	11.32 ± 0.694 (6)	10.47 ± 1.033 (6)	11.22 ± 2.206 (6)	10.95 ± 1.390 (6)	10.38 ± 0.677 (6)	10.78 ± 1.082 (6)
	21 [g]	10.23 ± 0.753 (6)	9.78 ± 0.508 (6)	9.58 ± 0.976 (5)	9.62 ± 0.823 (6)	9.80 ± 0.626 (6)	9.97 ± 0.686 (6)
Hematocrit (%)	7 [g1]	37.88 ± 2.504 (6)	34.80 ± 3.239 (6)	37.93 ± 8.823 (6)	37.25 ± 4.678 (6)	35.27 ± 2.060 (6)	35.90 ± 3.994 (6)
	21 [g]	35.68 ± 3.301 (6)	34.42 ± 2.252 (6)	33.80 ± 3.648 (5)	33.43 ± 3.248 (6)	34.42 ± 2.460 (6)	34.95 ± 3.210 (6)
MCV (fL)	7 [g]	62.38 ± 2.121 (6)	61.90 ± 2.156 (6)	64.93 ± 3.579 (6)	64.27 ± 2.717 (6)	63.43 ± 2.601 (6)	63.12 ± 3.947 (6)
	21 [g]	59.68 ± 2.503 (6)	57.67 ± 1.388 (6)	60.64 ± 2.534 (5)	60.40 ± 1.287 (6)	59.17 ± 0.963 (6)	59.80 ± 3.517 (6)
MCH (pg)	7 [g]	18.65 ± 0.720 (6)	18.62 ± 0.649 (6)	19.30 ± 0.369 (6)	18.90 ± 0.800 (6)	18.68 ± 0.857 (6)	18.95 ± 1.017 (6)
	21 [g]	17.13 ± 0.747 (6)	16.40 ± 0.746 (6)	17.18 ± 0.512 (5)	17.42 ± 0.422 (6)	16.88 ± 0.417 (6)	17.07 ± 0.706 (6)
MCHC (g/dL)	7 [g]	29.88 ± 0.366 (6)	30.08 ± 0.694 (6)	29.78 ± 1.111 (6)	29.42 ± 0.436 (6)	29.42 ± 0.588 (6)	30.08 ± 0.556 (6)
	21 [g]	28.72 ± 0.981 (6)	28.43 ± 0.689 (6)	28.32 ± 0.526 (5)	28.80 ± 0.669 (6)	28.52 ± 0.504 (6)	28.55 ± 0.873 (6)
Platelets (10^3 cells/µL)	7 [g]	338.8 ± 129.95 (6)	376.3 ± 96.99 (6)	406.3 ± 79.71 (6)	338.0 ± 97.17 (6)	363.7 ± 97.07 (6)	375.8 ± 172.88 (6)
	21 [g]	525.0 ± 128.14 (6)	473.3 ± 155.96 (6)	518.2 ± 106.23 (5)	507.0 ± 152.52 (6)	534.2 ± 59.15 (6)	505.2 ± 88.16 (6)
Absolute Reticulocyte (10^3 cells/µL)	7 [g]	164.40 ± 26.996 (6)	202.83 ± 79.008 (6)	193.85 ± 98.450 (6)	191.13 ± 83.548 (6)	185.34 ± 49.619 (6)	199.70 ± 56.779 (6)
	21 [g]	505.10 ± 128.983 (6)	522.23 ± 144.895 (6)	447.01 ± 118.419 (5)	489.42 ± 64.458 (6)	579.73 ± 120.025 (6)	560.36 ± 136.182 (6)
Neutrophils (10^3 cells/µL)	7 [g1]	2.972 ± 0.6130 (6)	2.580 ± 0.5956 (6)	4.105 ± 3.2263 (6)	4.035 ± 2.0612 (6)	2.460 ± 0.7959 (6)	3.078 ± 0.9762 (6)
	21 [g]	3.465 ±1.2166 (6)	2.887 ± 0.9044 (6)	2.930 ± 0.8489 (5)	3.033 ± 1.2156 (6)	3.322 ± 1.7464 (6)	3.120 ± 1.3319 (6)
Lymphocytes (10^3 cells/µL)	7 [g]	3.953 ± 1.3391 (6)	3.613 ± 1.0854 (6)	3.907 ± 1.6667 (6)	4.348 ± 0.8825 (6)	3.590 ± 0.5723 (6)	4.055 ± 0.4197 (6)
	21 [g]	6.032 ± 1.5573 (6)	5.138 ± 1.7954 (6)	5.080 ± 1.3370 (5)	5.318 ± 1.0343 (6)	4.898 ± 0.7903 (6)	6.683 ± 3.7236 (6)

Table 23. Hematology (Mean ± St Dev (n))

Parameter	Day	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Monocytes (10^3 cells/µL)	7 [g1]	0.250 ± 0.0802 (6)	0.228 ± 0.0542 (6)	0.340 ± 0.3211 (6)	0.307 ± 0.0952 (6)	0.295 ± 0.1247 (6)	0.325 ± 0.0638 (6)
	21 [g]	0.318 ± 0.1566 (6)	0.252 ± 0.1141 (6)	0.304 ± 0.1064 (5)	0.407 ± 0.1969 (6)	0.420 ± 0.2550 (6)	0.387 ± 0.3219 (6)
Leukocytes (10^3 cells/µL)	7 [g2]	0.118 ± 0.1251 (6)	0.112 ± 0.1192 (6)	0.085 ± 0.0850 (6)	0.095 ± 0.0843 (6)	0.057 ± 0.0493 (6)	0.110 ± 0.0555 (6)
	21 [g]	0.167 ± 0.1138 (6)	0.143 ± 0.1141 (6)	0.102 ± 0.1119 (5)	0.163 ± 0.1188 (6)	0.105 ± 0.0524 (6)	0.212 ± 0.0531 (6)
Erythrocytes (10^6 cells/µL)	7 [g2]	0.032 ± 0.0299 (6)	0.017 ± 0.0052 (6)	0.027 ± 0.0320 (6)	0.033 ± 0.0121 (6)	0.022 ± 0.0075 (6)	0.030 ± 0.0089 (6)
	21 [g]	0.065 ± 0.0493 (6)	0.045 ± 0.0362 (6)	0.040 ± 0.0381 (5)	0.037 ± 0.0250 (6)	0.030 ± 0.0268 (6)	0.142 ± 0.2160 (6)
Hemoglobin (g/dL)	7 [g]	0.110 ± 0.0438 (6)	0.100 ± 0.0322 (6)	0.088 ± 0.0397 (6)	0.118 ± 0.0605 (6)	0.067 ± 0.0301 (6)	0.075 ± 0.0288 (6)
	21 [g]	0.082 ± 0.0618 (6)	0.090 ± 0.0322 (6)	0.070 ± 0.0592 (5)	0.085 ± 0.0748 (6)	0.098 ± 0.0752 (6)	0.127 ± 0.0516 (6)
Hematocrit (%)	7 [g]	16.53 ± 0.339 (6)	17.35 ± 0.804 (6)	16.80 ± 0.921 (6)	16.62 ± 1.160 (6)	16.98 ± 1.350 (6)	16.47 ± 0.747 (6)
	21 [g]	17.97 ± 0.612 (6)	18.67 ± 0.480 (6)	18.20 ± 0.797 (5)	18.17 ± 0.388 (6)	18.63 ± 0.327 (6)	18.53 ± 0.999 (6)

Abbreviations for Hematology Parameters: MCV – Mean Corpuscular Volume; MCH – Mean Corpuscular Hemoglobin; MCHC – Mean Corpuscular Hemoglobin Concentration; RDW – Red Blood Cell Distribution Width

[g] – ANOVA & Dunnett (Log)

[g1] – ANOVA & Dunnett

[g2] – Kruskal-Wallis & Dunn

Table 24. Coagulation Parameters (Mean ± St Dev (n))

Parameter	Day	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
APTT (sec)	7 [g]	13.47 ± 1.060 (6)	13.65 ± 0.742 (6)	13.88 ± 1.109 (6)	13.08 ± 0.708 (6)	13.53 ± 0.905 (6)	13.00 ± 1.243 (6)
	21 [g]	13.30 ± 0.974 (6)	13.47 ± 0.774 (6)	14.28 ± 1.221 (5)	13.10 ± 1.231 (6)	13.70 ± 0.894 (6)	13.90 ± 1.147 (6)
Prothrombin Time (sec)	7 [g]	12.60 ± 0.379 (6)	12.77 ± 0.314 (6)	13.37 ± 0.344 (6)[b]	12.83 ± 0.372 (6)	12.92 ± 0.462 (6)	13.17 ± 0.308 (6)
	21 [g]	12.47 ± 0.423 (6)	12.50 ± 0.261 (6)	12.72 ± 0.356 (5)	12.62 ± 0.483 (6)	12.75 ± 0.657 (6)	12.90 ± 0.354 (5)
Fibrinogen (mg/dL)	7 [g1]	168.7 ± 24.69 (6)	160.8 ± 7.57 (6)	168.0 ± 50.46 (6)	159.0 ± 16.80 (6)	147.5 ± 27.08 (6)	191.2 ± 120.91 (6)
	21 [g]	188.5 ± 14.24 (6)	172.0 ± 32.70 (6)	161.2 ± 18.79 (5)	194.5 ± 47.55 (6)	186.2 ± 27.41 (6)	184.8 ± 30.24 (5)

Abbreviations for Coagulation Parameters: APTT – Activated Partial Thromboplastin Time
[g] – ANOVA & Dunnett
[g1] – ANOVA & Dunnett (Log)
[g2] – Kruskal-Wallis & Dunn
b = $p < 0.01$

Table 25. Clinical Chemistry (Mean ± St Dev (n))							
Parameter	**Day**	**Male**			**Female**		
		0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
Sodium (mEq/L)	7 [g]	138.7 ± 3.59 (6)	138.9 ± 2.71 (6)	140.4 ± 5.70 (6)	139.6 ± 3.23 (6)	138.6 ± 2.12 (6)	140.8 ± 1.11 (6)
	21 [g]	143.8 ± 1.33 (6)	144.2 ± 3.03 (6)	142.9 ± 1.21 (5)	141.8 ± 2.00 (6)	143.6 ± 1.59 (6)	144.8 ± 1.94 (6)[a]
Potassium (mEq/L)	7 [g1]	6.64 ± 0.531 (6)	6.52 ± 0.723 (6)	6.56 ± 1.884 (6)	6.60 ± 0.607 (6)	6.63 ± 0.632 (6)	6.51 ± 0.704 (6)
	21 [g]	6.77 ± 0.506 (6)	6.70 ± 0.424 (6)	6.44 ± 0.421 (5)	6.20 ± 0.734 (6)	6.32 ± 0.459 (6)	6.67 ± 0.527 (6)
Chloride (mEq/L)	7 [g2]	101.5 ± 3.11 (6)	102.8 ± 3.75 (6)	107.7 ± 12.31 (6)	103.3 ± 2.42 (6)	102.1 ± 2.54 (6)	103.0 ± 1.76 (6)
	21 [g2]	105.8 ± 1.41 (6)	105.4 ± 1.99 (6)	104.4 ± 0.57 (5)	104.7 ± 1.29 (6)	105.2 ± 1.97 (6)	105.7 ± 1.10 (6)
Calcium (mg/dL)	7 [g2]	10.86 ± 0.303 (6)	10.92 ± 0.511 (6)	10.85 ± 1.063 (6)	10.85 ± 0.619 (6)	11.07 ± 0.575 (6)	11.28 ± 0.223 (6)
	21 [g]	10.87 ± 0.234 (6)	11.03 ± 0.296 (6)	10.85 ± 0.093 (5)	10.52 ± 0.268 (6)	10.84 ± 0.235 (6)	10.92 ± 0.197 (6)a
Phosphorus (mg/dL)	7 [g1]	8.32 ± 0.676 (6)	8.08 ± 0.598 (6)	8.46 ± 1.938 (6)	8.74 ± 1.017 (6)	8.39 ± 0.913 (6)	8.51 ± 0.551 (6)
	21 [g]	10.31 ± 0.861 (6)	10.19 ± 1.224 (6)	10.19 ± 0.563 (5)	10.21 ± 1.096 (6)	10.26 ± 0.606 (6)	10.61 ± 0.794 (6)
ALP (U/L)	7 [g1]	444.0 ± 182.21 (6)	886.5 ± 704.82 (6)	509.4 ± 266.21 (6)	491.3 ± 193.73 (6)	618.9 ± 162.27 (6)	457.7 ± 156.73 (6)
	21 [g2]	486.6 ± 64.21 (6)	498.6 ± 142.62 (6)	471.8 ± 115.17 (5)	623.3 ± 259.77 (6)	618.2 ± 175.40 (6)	412.4 ± 54.82 (6)
Total Bilirubin (mg/dL)	7 [g1]	0.22 ± 0.067 (6)	0.29 ± 0.187 (6)	0.18 ± 0.040 (6)	0.23 ± 0.097 (6)	0.19 ± 0.087 (6)	0.18 ± 0.070 (6)
	21 [g]	0.14 ± 0.026 (6)	0.15 ± 0.021 (6)	0.15 ± 0.030 (5)	0.15 ± 0.016 (6)	0.15 ± 0.008 (6)	0.15 ± 0.034 (6)
GGT (U/L)	7 [g2]	27.9 ± 14.68 (6)	31.6 ± 12.08 (6)	35.8 ± 3.32 (6)	24.5 ± 8.52 (6)	25.3 ± 6.49 (6)	29.6 ± 6.32 (6)
	21 [g]	21.0 ± 8.72 (6)	24.4 ± 8.30 (6)	26.7 ± 5.37 (5)	18.8 ± 6.24 (6)	20.9 ± 4.09 (6)	30.9 ± 19.64 (6)
AST (U/L)	7 [g1]	62.0 ± 62.10 (6)	32.8 ± 7.34 (6)	31.3 ± 15.78 (6)	32.6 ± 2.92 (6)	34.4 ± 13.64 (6)	36.8 ± 11.21 (6)
	21 [g]	31.8 ± 5.46 (6)	33.9 ± 5.78 (6)	36.5 ± 7.41 (5)	42.0 ± 18.80 (6)	32.9 ± 6.65 (6)	50.8 ± 22.62 (6)
ALT (U/L)	7 [g1]	28.0 ± 10.52 (6)	20.9 ± 2.76 (6)	23.1 ± 3.31 (6)	28.7 ± 4.02 (6)	24.2 ± 4.03 (6)	23.3 ± 7.05 (6)
	21 [g]	23.3 ± 5.21 (6)	22.7 ± 4.23 (6)	25.1 ± 2.29 (5)	24.5 ± 5.90 (6)	22.7 ± 5.04 (6)	24.2 ± 4.56 (6)
SDH (U/L)	7 [g]	3.77 ± 3.288 (3)	4.68 ± 1.024 (4)	1.47 ± 0.603 (3)	0.70 ± - (1)[n]	1.18 ± 0.512 (4)[n]	2.68 ± 1.546 (4)[n]
	21 [I]	1.20 ± 0.707 (2)[n]	1.28 ± 0.631 (6)[n]	2.07 ± 1.159 (3)[n]	1.10 ± 0.141 (2)[n]	2.18 ± 1.668 (4)[n]	1.33 ± 0.737 (3)[n]
Urea Nitrogen (mg/dL)	7 [g1]	9.3 ± 2.22 (6)	9.6 ± 5.59 (6)	28.0 ± 49.70 (6)	9.9 ± 3.34 (6)	5.9 ± 2.57 (6)	5.7 ± 3.02 (6)
	21 [g]	6.4 ± 0.86 (6)	6.3 ± 1.26 (6)	5.0 ± 1.03 (5)	6.9 ± 0.94 (6)	5.2 ± 1.24 (6)[a]	5.3 ± 1.02 (6)[a]

Table 25. Clinical Chemistry (Mean ± St Dev (n))							
Parameter	**Day**	**Male**			**Female**		
		0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
Creatinine (mg/dL)	7 [g2]	0.51 ± 0.132 (6)	0.53 ± 0.035 (6)	0.87 ± 0.892 (6)	0.46 ± 0.077 (6)	0.52 ± 0.109 (6)	0.53 ± 0.046 (6)
	21 [g]	0.59 ± 0.082 (6)	0.61 ± 0.103 (6)	0.57 ± 0.081 (5)	0.54 ± 0.107 (6)	0.55 ± 0.107 (6)	0.55 ± 0.050 (6)
Total Protein (g/dL)	7 [g2]	4.81 ± 0.205 (6)	4.64 ± 0.270 (6)	5.00 ± 1.039 (6)	4.75 ± 0.288 (6)	4.82 ± 0.219 (6)	4.61 ± 0.642 (6)
	21 [g1]	4.12 ± 0.479 (6)	3.92 ± 0.201 (6)	4.20 ± 0.413 (5)	4.22 ± 0.424 (6)	4.25 ± 0.305 (6)	4.38 ± 0.436 (6)
Albumin (g/dL)	7 [g2]	1.71 ± 0.108 (6)	1.71 ± 0.158 (5)	1.86 ± 0.520 (6)	1.70 ± 0.093 (5)	1.66 ± 0.136 (5)	1.72 ± 0.081 (5)
	21 [g]	2.22 ± 0.179 (6)	2.25 ± 0.200 (6)	2.36 ± 0.108 (5)	2.25 ± 0.122 (6)	2.40 ± 0.295 (6)	2.51 ± 0.186 (6)
Globulin (g/dL)	7 [g]	3.10 ± 0.256 (6)	2.99 ± 0.163 (5)	3.14 ± 0.565 (6)	3.10 ± 0.366 (5)	3.21 ± 0.157 (5)	3.04 ± 0.556 (5)
	21 [g]	1.90 ± 0.510 (6)	1.68 ± 0.154 (6)	1.84 ± 0.369 (5)	1.97 ± 0.464 (6)	1.85 ± 0.230 (6)	1.87 ± 0.353 (6)
Albumin/Globulin	7 [g]	0.56 ± 0.071 (6)	0.57 ± 0.049 (5)	0.59 ± 0.087 (6)	0.56 ± 0.096 (5)	0.52 ± 0.050 (5)	0.58 ± 0.128 (5)
	21 [g]	1.24 ± 0.323 (6)	1.36 ± 0.209 (6)	1.33 ± 0.289 (5)	1.21 ± 0.337 (6)	1.32 ± 0.251 (6)	1.38 ± 0.237 (6)
Triglyceride (mg/dL)	7 [g]	30.1 ± 6.20 (6)	48.1 ± 17.59 (6)	43.2 ± 24.31 (6)	44.6 ± 12.95 (6)	42.2 ± 9.29 (6)	49.3 ± 19.51 (6)
	21 [g2]	17.7 ± 5.17 (6)	32.2 ± 13.35 (6)	16.2 ± 2.21 (5)	22.1 ± 10.18 (6)	16.1 ± 3.66 (6)	18.8 ± 6.79 (6)
Cholesterol (mg/dL)	7 [g1]	78.4 ± 8.85 (6)	79.8 ± 15.48 (6)	94.3 ± 52.68 (6)	85.9 ± 13.25 (6)	80.7 ± 14.82 (6)	72.4 ± 8.01 (6)
	21 [g]	67.2 ± 6.73 (6)	65.4 ± 7.64 (6)	69.1 ± 6.28 (5)	75.3 ± 7.45 (6)	77.4 ± 9.40 (6)	70.0 ± 10.50 (6)
LDL Cholesterol (mg/dL)	7 [g1]	29.8 ± 3.36 (6)	30.5 ± 8.21 (6)	44.9 ± 37.76 (6)	32.0 ± 6.34 (6)	29.2 ± 7.81 (6)	27.0 ± 2.48 (6)
	21 [g]	28.4 ± 4.51 (6)	26.1 ± 4.89 (6)	29.1 ± 2.23 (5)	35.0 ± 6.44 (6)	32.2 ± 7.78 (6)	30.7 ± 6.21 (6)
Glucose (mg/dL)	7 [g]	130.6 ± 22.09 (6)	116.7 ± 20.81 (6)	113.6 ± 16.47 (6)	114.1 ± 12.21 (6)	126.9 ± 17.22 (6)	133.4 ± 7.42 (6)
	21 [g1]	146.0 ± 16.47 (6)	145.5 ± 5.91 (6)	140.1 ± 7.24 (5)	138.0 ± 10.55 (6)	141.7 ± 8.75 (6)	141.3 ± 4.83 (6)
GLDH (U/L)	7 [g1]	4.3 ± 4.89 (6)	2.8 ± 3.06 (6)	2.0 ± 0.89 (6)	2.5 ± 0.55 (6)	1.8 ± 0.98 (6)	2.2 ± 0.75 (6)
	21 [g]	1.3 ± 0.52 (6)	1.3 ± 0.52 (6)	1.8 ± 0.84 (5)	2.2 ± 1.17 (6)	1.3 ± 0.52 (6)	1.7 ± 0.82 (6)

Abbreviations for Coagulation Parameters: GGT - Gamma Glutamyltransferase; AST - Aspartate Aminotransferase; ALT - Alanine Aminotransferase; ALP - Alkaline Phosphatase; GLDH - Glutamate Dehydrogenase; SDH - Sorbitol Dehydrogenase; LDL - Low Density Lipoprotein

[g] – ANOVA & Dunnett

[g1] – Kruskal-Wallis & Dunn

[I] – n = Inappropriate for statistics

a = $p < 0.01$

Table 26. Urinalysis (Mean ± St. Dev (n))

Parameter	Day	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Volume (mL)	22 [g]	20.8 ± 8.61 (6)	14.2 ± 9.17 (6)	20.2 ± 17.40 (5)	19.0 ± 24.71 (4)	21.0 ± 14.35 (6)	37.5 ± 21.62 (6)
Specific Gravity	22 [g]	1.0130 ± 0.00429 (6)	1.0143 ± 0.00403 (6)	1.0126 ± 0.00288 (5)	1.0140 ± 0.00400 (5)	1.0112 ± 0.00232 (6)	1.0122 ± 0.00204 (6)
pH	22 [I]	8.50 ± - $(1)^n$	-	-	NA	NA	NA

[g] – ANOVA & Dunnett
[I] – n = Inappropriate for statistics

Organ Weights: Absolute and/or relative cecum weights increased dose-dependently in males and females at ≥5.75 g/L with statistical significance limited to relative cecum/body weight percentage in males at 8.0 g/L (Table 23). No microscopic correlates were observed to account for the increased cecum weights.

Table 27. Summary of Large Intestinal Weight Data – Scheduled/Terminal Euthanasia (Day 22)

	Male			Female		
Dose (mg/kg/day)	**0 g/L**	**5.75 g/L**	**8.0 g/L**	**0 g/L**	**5.75 g/L**	**8.0 g/L**
No. animals per group	**6**	**6**	**5**	**6**	**6**	**6**
Large intestine, cecum (No. weighed)	(6)	(6)	(5)	(6)	(6)	(6)
Absolute value (g)	6.1265	+14.6	+37.3	4.5867	+46.6	+65.5
Relative to body weight	0.11151	+22.4	**+31.9**	0.08775	+42.8	+56.0
Relative to brain weight	0.13264	+17.4	+40.0	0.10045	+45.7	+66.5
Large intestine, colon (No. weighed)	(6)	(6)	(5)	(6)	(6)	(6)
Absolute value (g)	39.3055	+10.6	+27.9	41.1590	+16.1	+19.8
Relative to body weight	0.71070	+20.4	**+28.8**	0.79148	+12.9	+13.3
Relative to brain weight	0.84944	+13.4	+30.1	0.89771	+14.6	+20.5
Large intestine, rectum (No. weighed)	(6)	(6)	(5)	(6)	(6)	(6)
Absolute value (g)	14.1277	-12.7	-31.2	12.3943	+4.4	-23.8
Relative to body weight	0.24747	-2.9	-29.9	0.24757	-3.8	-30.6
Relative to brain weight	0.30346	-10.0	-29.8	0.27318	+0.8	-24.1

All values in dosed groups are expressed as percent difference of control group means.
Based upon statistical analysis of group means, values highlighted in bold are significantly different from control group - $p < 0.05$; refer to data tables for actual significance levels and tests used.

Increased absolute and/or relative colon weights were present in males in a dose dependent manner at ≥5.75 g/L with statistical significance reached for/limited to relative colon/body weight percentage in males at 8.0 g/L. Absolute and relative colon weights were slightly higher in females at ≥5.75 g/L in comparison to concurrent control females; however, the weight changes lacked dose dependency and were comparable in females at 5.75 g/L and 8.0 g/L. The increased colon weights lacked microscopic correlates.

Decreased absolute and/or relative rectum weights were present in males and females at 8.0 g/L; there were no microscopic correlates to account for the rectal weight changes. The absolute rectal weight of one control male was much higher than all other animals and likely skewed weight comparisons.

A summary of the other absolute and relative organ weights is shown in Table 24. Other differences in organ weight parameters were attributed to normal biologic variation. These differences had no patterns, trends, or correlating data to suggest these differences were test article related.

Table 28. Absolute and Relative Organ Weights

Organ	Parameter	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Body [g] Weight (kg)	Mean ± SD (n)	5.52 ± 0.760 (6)	5.12 ± 0.950 (6)	5.58 ± 1.262 (5)	5.23 ± 0.940 (6)	5.37 ± 0.698 (6)	5.48 ± 0.674 (6)
	%Diff	-	-7.3	1.1	-	2.5	4.8
Brain [g] (g)	Mean ± SD (n)	46.4342 ±2.27159 (6)	45.2262 ± 1.76793 (6)	45.4914 ± 1.50483 (5)	45.6850 ± 1.74550 (6)	46.4005 ± 2.51887 (6)	45.5953 ± 1.35595 (6)
	%Diff	-	-2.6	-2.0	-	1.6	-0.2
Brain/BWt [g] (%)	Mean ± SD (n)	0.85519 ± 0.126414 (6)	0.90982 ± 0.171769 (6)	0.84744 ± 0.183059 (5)	0.89216 ± 0.129758 (6)	0.87490 ± 0.105879 (6)	0.84241 ± 0.109949 (6)
	%Diff	-	6.4	-0.9	-	-1.9	-5.6
Heart [g] (g)	Mean ± SD (n)	40.9493 ± 3.96562 (6)	36.5488 ± 6.44242 (6)	42.6080 ± 9.19517 (5)	38.7503 ± 7.32526 (6)	38.4490 ± 3.34122 (6)	43.1478 ± 3.99862 (6)
	%Diff	-	-10.7	4.1	-	-0.8	11.3
Heart/BWt [g] (%)	Mean ± SD (n)	0.74735 ± 0.060102 (6)	0.71732 ± 0.062523 (6)	0.76913 ± 0.088339 (5)	0.73978 ± 0.023888 (6)	0.72036 ± 0.046846 (6)	0.79108 ± 0.062033 (6)
	%Diff	-	-4.0	2.9	-	-2.6	6.9
Heart/BrWt [g] (ratio)	Mean ± SD (n)	0.88451 ± 0.104771 (6)	0.80986 ± 0.150592 (6)	0.93454 ± 0.185407 (5)	0.84451 ± 0.130547 (6)	0.83031 ± 0.078506 (6)	0.94742 ± 0.097587 (6)
	%Diff	-	-8.4	5.7	-	-1.7	12.2
Kidneys [g] (g)	Mean ± SD (n)	52.3180 ± 9.79544 (6)	45.0632 ± 10.72428 (6)	51.0532 ± 12.54261 (5)	49.0230 ± 12.00576 (6)	55.6135 ± 12.48572 (6)	52.6713 ± 9.52917 (6)
	%Diff	-	-13.9	-2.4	-	13.4	7.4
Kidneys/BWt [g] (%)	Mean ± SD (n)	0.94807 ± 0.103724 (6)	0.87523 ± 0.086778 (6)	0.91439 ± 0.078706 (5)	0.92758 ± 0.079143 (6)	1.04371 ± 0.255006 (6)	0.96077 ± 0.115022 (6)
	%Diff	-	-7.7	-3.6	-	12.5	3.6

Table 28. Absolute and Relative Organ Weights

Organ	Parameter	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Kidneys/BrWt [g] (ratio)	Mean ± SD (n)	1.13270 ± 0.240726 (6)	0.99983 ± 0.250956 (6)	1.12057 ± 0.265069 (5)	1.06670 ± 0.228170 (6)	1.19551 ± 0.241434 (6)	1.15669 ± 0.218574 (6)
	%Diff	-	-11.7	-1.1	-	12.1	8.4
Large intes. [g] Cecum (g)	Mean ± SD (n)	6.1265 ± 0.90220 (6)	7.0180 ± 1.69637 (6)	8.4092 ± 3.30331 (5)	4.5867 ± 2.03619 (6)	6.7233 ± 3.06418 (6)	7.5897 ± 2.14859 (6)
	%Diff	-	14.6	37.3	-	46.6	65.5
Large intes, [g2] cecum/BWt (%)	Mean ± SD (n)	0.11151 ± 0.013569 (6)	0.13643 ± 0.010787 (6)	0.14705 ± 0.039849 (5)[a]	0.08775 ± 0.033035 (6)	0.12527 ± 0.054388 (6)	0.13692 ± 0.029630 (6)
	%Diff	-	22.4	31.9	-	42.8	56.0
Large intes, [g] cecum/BrWt (ratio)	Mean ± SD (n)	0.13264 ± 0.024087 (6)	0.15574 ± 0.040113 (6)	0.18564 ± 0.073885 (5)	0.10045 ± 0.043463 (6)	0.14631 ± 0.070798 (6)	0.16729 ± 0.049674 (6)
	%Diff	-	17.4	40.0	-	45.7	66.5
Large intes. [g] Colon (g)	Mean ± SD (n)	39.3055 ± 6.69121 (6)	43.4543 ± 7.98932 (6)	50.2732 ± 10.93027 (5)	41.1590 ± 6.57621 (6)	47.7657 ± 9.12388 (6)	49.2982 ± 7.75995 (6)
	%Diff	-	10.6	27.9	-	16.1	19.8
Large intes, [g] colon/BWt (%)	Mean ± SD (n)	0.71070 ± 0.040866 (6)	0.85587 ± 0.130278 (6)	0.91509 ± 0.175353 (5)[a]	0.79148 ± 0.083759 (6)	0.89336 ± 0.155568 (6)	0.89678 ± 0.052351 (6)
	%Diff	-	20.4	28.8	-	12.9	13.3
Large intes, [g] colon/BrWt (ratio)	Mean ± SD (n)	0.84944 ± 0.158164 (6)	0.96353 ± 0.186606 (6)	1.10531 ± 0.242526 (5)	0.89771 ± 0.115127 (6)	1.02911 ± 0.183401 (6)	1.08189 ± 0.173931 (6)
	%Diff	-	13.4	30.1	-	14.6	20.5
Large intes. [g] Rectum (g)	Mean ± SD (n)	14.1277 ± 7.89143 (6)	12.3357 ± 7.31793 (6)	9.7204 ± 2.72675 (5)	12.3943 ± 3.25852 (6)	12.9422 ± 7.63456 (6)	9.4415 ± 1.66453 (6)
	%Diff	-	-12.7	-31.2	-	4.4	-23.8

Table 28. Absolute and Relative Organ Weights

Organ	Parameter	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Large intes, [g] rectum/BWt (%)	Mean ± SD (n)	0.24747 ± 0.104625 (6)	0.24031 ± 0.133841 (6)	0.17353 ± 0.022982 (5)	0.24757 ± 0.093317 (6)	0.23808 ± 0.125050 (6)	0.17172 ± 0.018482 (6)
	%Diff	-	-2.9	-29.9	-	-3.8	-30.6
Large intes, [g] rectum/BrWt (ratio)	Mean ± SD (n)	0.30346 ± 0.165604 (6)	0.27319 ± 0.165705 (6)	0.21312 ± 0.056898 (5)	0.27318 ± 0.080039 (6)	0.27524 ± 0.150411 (6)	0.20743 ± 0.038094 (6)
	%Diff	-	-10.0	-29.8	-	0.8	-24.1
Liver w/ [g] Gallbladder (g)	Mean ± SD (n)	181.5603 ± 22.06378 (6)	170.0287 ± 29.61167 (6)	189.6808 ± 36.37935 (5)	186.0467 ± 30.35304 (6)	182.7653 ± 28.28351 (6)	189.5793 ± 22.68564 (6)
	%Diff	-	-6.4	4.5	-	-1.8	1.9
Liver w/ GB [g] /BWt (%)	Mean	3.30938 ± 0.317106 (6)	3.33397 ± 0.231707 (6)	3.42467 ± 0.244156 (5)	3.58558 ± 0.407498 (6)	3.40645 ± 0.336352 (6)	3.49212 ± 0.519496 (6)
	%Diff	-	0.7	3.5	-	-5.0	-2.6
Liver w/ GB [g] /BrWt (ratio)	Mean	3.91412 ± 0.468559 (6)	3.76490 ± 0.679597 (6)	4.16844 ± 0.765231 (5)	4.06531 ± 0.576787 (6)	3.94377 ± 0.611197 (6)	4.16009 ± 0.508501 (6)
	%Diff	-	-3.8	6.5	-	-3.0	2.3
Small intes. [g] Duodenum (g)	Mean ± SD (n)	62.3568 ± 13.72859 (6)	56.8028 ± 15.81976 (6)	61.9216 ± 11.33367 (5)	61.2420 ± 15.35857 (6)	63.3058 ± 13.22122 (6)	62.9915 ± 16.85156 (6)
	%Diff	-	-8.9	-0.7	-	3.4	2.9
Small intest [g] duodenum/BWt (%)	Mean ± SD (n)	1.12189 ± 0.108449 (6)	1.09742 ± 0.181502 (6)	1.12029 ± 0.119668 (5)	1.16656 ± 0.184777 (6)	1.17408 ± 0.159768 (6)	1.14063 ± 0.159768 (6)
	%Diff	-	-2.2	-0.1	-	0.6	-2.2
Small intest [g] duoden/BrWt (ratio)	Mean ± SD (n)	1.34402 ± 0.289943 (6)	1.25607 ± 0.356362 (6)	1.36372 ± 0.258269 (5)	1.33719 ± 0.315534 (6)	1.36546 ± 0.283729 (6)	1.38965 ± 0.401617 (6)
	%Diff	-	-6.5	1.5	-	2.1	3.9

Table 28. Absolute and Relative Organ Weights							
Organ	**Parameter**	**Male**			**Female**		
		0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
Small intes. [g] Ileum (g)	Mean ± SD (n)	68.8393 ± 17.35510 (6)	55.2483 ± 14.07396 (6)	57.5178 ± 14.42920 (5)	62.0133 ± 10.82514 (6)	71.6380 ± 12.52760 (6)	62.2550 ± 9.70701 (6)
	%Diff	-	-19.7	-16.4	-	15.5	0.4
Small intest [g] ileum/BWt (%)	Mean ± SD (n)	1.23327 ± 0.144446 (6)	1.08437 ± 0.232932 (6)	1.07773 ± 0.350339 (5)	1.20846 ± 0.247090 (6)	1.34621 ± 0.265065 (6)	1.13674 ± 0.136810 (6)
	%Diff	-	-12.1	-12.6	-	11.4	-5.9
Small intest [g] ileum/BrWt (ratio)	Mean ± SD (n)	1.48238 ± 0.361087 (6)	1.22447 ± 0.321891 (6)	1.27037 ± 0.336343 (5)	1.35814 ± 0.234962 (6)	1.54242 ± 0.252009 (6)	1.36757 ± 0.224347 (6)
	%Diff	-	-17.4	-14.3	-	13.6	0.7
Small intes. [g] Jejunum (g)	Mean ± SD (n)	107.1463 ± 16.80541 (6)	98.0702 ± 19.11400 (6)	114.0058 ± 26.51077 (5)	107.9805 ± 18.97667 (6)	100.4538 ± 29.88983 (6)	104.8582 ± 29.37227 (6)
	%Diff	-	-8.5	6.4	-	-7.0	-2.9
Small intest [g] jejunum/BWt (%)	Mean ± SD (n)	1.93874 ± 0.099756 (6)	1.91520 ± 0.131229 (6)	2.05068 ± 0.232574 (5)	2.07913 ± 0.275015 (6)	1.85539 ± 0.480064 (6)	1.88605 ± 0.362797 (6)
	%Diff	-	-1.2	5.8	-	-10.8	-9.3
Small intest [g] jejunum/BrWt (ratio)	Mean ± SD (n)	2.31214 ± 0.375855 (6)	2.17208 ± 0.437661 (6)	2.50393 ± 0.549809 (5)	2.36072 ± 0.377940 (6)	2.16026 ± 0.630711 (6)	2.30453 ± 0.651475 (6)
	%Diff	-	-6.1	8.3	-	-8.5	-2.4
Spleen [g] (g)	Mean ± SD (n)	14.4430 ± 3.45672 (6)	12.7775 ± 4.19351 (6)	18.7658 ± 6.09529 (5)	12.8693 ± 5.27034 (6)	15.0110 ± 5.70000 (6)	16.2663 ± 5.60274 (6)
	%Diff	-	-11.5	29.9	-	16.6	26.4
Spleen/BWt [g] (%)	Mean ± SD (n)	0.26720 ± 0.078382 (6)	0.25602 ± 0.103527 (6)	0.33793 ± 0.095440 (5)	0.24699 ± 0.103373 (6)	0.28746 ± 0.131917 (6)	0.29510 ± 0.084724 (6)
	%Diff	-	-4.2	26.5	-	16.4	19.5

Table 28. Absolute and Relative Organ Weights

Organ	Parameter	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Spleen/BrWt [g] (ratio)	Mean ± SD (n)	0.31408 ± 0.089031 (6)	0.28375 ± 0.095445 (6)	0.41103 ± 0.130776 (5)	0.28170 ± 0.119076 (6)	0.32363 ± 0.123027 (6)	0.35755 ± 0.126140 (6)
	%Diff	-	-9.7	30.9	-	14.9	26.9
Thymus [g] (g)	Mean ± SD (n)	17.3868 ± 3.53791 (6)	15.5063 ± 5.41095 (6)	19.2192 ± 7.80399 (5)	24.8100 ± 15.55090 (6)	17.2813 ± 3.74387 (6)	19.6007 ± 3.55849 (6)
	%Diff	-	-10.8	10.5	-	-30.3	-21.0
Thymus/BWt [g] (%)	Mean ± SD (n)	0.32234 ± 0.085145 (6)	0.29737 ± 0.070755 (6)	0.33587 ± 0.069145 (5)	0.48098 ± 0.326427 (6)	0.32454 ± 0.072612 (6)	0.36039 ± 0.075225 (6)
	%Diff	-	-7.7	4.2	-	-32.5	-25.1
Thymus/BrWt [g] (ratio)	Mean ± SD (n)	0.37726 ± 0.089538 (6)	0.34337 ± 0.122475 (6)	0.42286 ± 0.173968 (5)	0.53839 ± 0.334214 (6)	0.37489 ± 0.091726 (6)	0.42956 ± 0.074522 (6)
	%Diff	-	-9.0	12.1	-	-30.4	-20.2
Thyroid [g] (g)	Mean ± SD (n)	0.8625 ± 0.15958 (6)	0.6395 ± 0.20366 (6)	0.8084 ± 0.17602 (5)	0.7060 ± 0.17182 (6)	0.7380 ± 0.09158 (6)	0.6490 ± 0.12372 (6)
	%Diff	-	-25.9	-6.3	-	4.5	-8.1
Thyroid gl/ [g] BWt (%)	Mean ± SD (n)	0.01609 ± 0.004537 (6)	0.01273 ± 0.003993 (6)	0.01461 ± 0.001742 (5)	0.01359 ± 0.002669 (6)	0.01391 ± 0.002210 (6)	0.01192 ± 0.002474 (6)
	%Diff	-	-20.9	-9.2	-	2.4	-12.3
Thyroid [g] gl/BrWt (ratio)	Mean ± SD (n)	0.01868 ± 0.003998 (6)	0.01413 ± 0.004467 (6)	0.01778 ± 0.003942 (5)	0.01543 ± 0.003502 (6)	0.01586 ± 0.001229 (6)	0.01424 ± 0.002766 (6)
	%Diff	-	-24.4	-4.8	-	2.8	-7.7

Abbreviations: BrWt - brain weight; BWt - body weight; duoden – duodenum; GB – gallbladder; gl – gland; intes/intest – intestine; w/ - with

[g] – ANOVA & Dunnett

[g1] – ANOVA & Dunnett (Log)

[g2] – Kruskal-Wallis & Dunn

A = $p < 0.05$

Histology: There were no Oligosaccharide Blend-related microscopic findings. With the exception of incidental mucosal gland dilation/inflammation, subacute inflammation, bacteria (gram negative bacilli) and/or goblet cell hypertrophy/hyperplasia and increased mucus in the gastrointestinal tract of one male at 8 g/L (Animal No. 3001), which was euthanized in extremis on Day 7, there were no meaningful differences in the gastrointestinal tract of treated animals in comparison to concurrent control animals.

All other microscopic observations were incidental and/or of the type occasionally observed in young swine (Glastonbury et al. 1977; Hamir 1980; Liu et al. 2005). All observations were of low incidence, lacked dose response, and/or occurred in concurrent control animals.

4. Discussion

Daily dietary administration of Oligosaccharide Blend in ProNurse® specialty milk replacer formula to neonatal piglets for 3 weeks following birth at concentrations of 5.75 or 8.0 g/L was well tolerated and did not produce adverse effects on their growth and development. This observation was based on a lack of adverse findings on body weight and food efficiency. 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. Organ weight changes were limited to increased cecum weights in males and females at ≥5.75 g/L, increased colon weights in males at ≥5.75 g/L, and decreased rectum weights in males and females at 8.0 g/L, but these changes were not considered adverse as there were no microscopic correlates. Additionally, studies have shown that nondigestible oligosaccharides (such as inulin and galactooligosaccharides) increase microbial fermentation and result in the production of osmotically active by-products, for example, short-chain fatty acids, which can cause soft stools and colon and cecal weight increase/enlargement (Aufreiter et al. 2011; Kruger et al. 2017). No adverse findings in gross or histopathology were noted.

E. CLINICAL STUDIES

As summarized in GRN 833, no clinical studies conducted with LNT have been published. Additionally, a search of PubMed on February 24, 2020 using the search terms “lacto-N-tetratose” and “clinical” yielded no new clinical studies.

F. ALLERGENICITY

Allergens, by definition, are antigens that are recognized by IgE antibodies and provoke IgE-mediated hypersensitivity responses (Aalberse, 2000). Most allergens are proteins or glycoproteins (Radauer et al., 2008; Sicherer and Sampson, 2010), although there have been a limited number of reports of allergic reactions to carbohydrates (Franck et al., 2005; Chiang et al., 2012; Commins et al., 2011). Additionally, allergic reactions to human milk have not been reported. Importantly, genetically engineered strains of *E. coli* BL21(DE3) have been safely used in the production of food and pharmaceutical ingredients (see Section VI.A) and product specifications control the level of protein derived from *JBT-LNT* in the finished ingredient (see Section II.G). Moreover, the genes used to engineer *JBT-LNT* are not derived from major allergens and full-length FASTA alignments of amino acid sequences of the genes used to engineer JBT-LNT and version 19 of the AllergenOnline Database maintained by the University of Nebraska – Lincoln showed that cross-reactivity with known allergens (≥ 50% identity) is not expected (Table 29). Thus, although the protein specification does not completely eliminate the possibility that consumers of Jennewein's LNT-containing ingredient may be exposed to the protein residues derived from the production organism (specification of ≤ 0.01 % protein), allergic reactions resulting from the exposure to theoretically possible protein residues derived from *JBT-LNT* in the finished ingredient are not expected.

Table 29. Percent Identity of the Genetic Manipulations in *JBT-LNT* with Known Allergens

Function	Origin of the gene	% Identity*
Lactose permease	*E. coli* K12	27.4
UDP-galactose-4-epimerase	*E. coli* K12	30.7
Galactokinase	*E. coli* K12	None
Galactose mutarotase	*E. coli* K12	26.9
Galactosyltransferase	*E. coli* K12	24.0
UTP-glucose-1-phosphate uridyltransferase	*E. coli* K12	None
β-1,3-*N*-acetylglucosaminyltransferase	*Neisseria meningitidis*	≤ 25.9
β-1,3-galactosyltransferase	*Salmonella enterica*	22.4
Glutamine fructose 6-phosphate aminotransferase	*E. coli* K12	23.2
Antibiotic Resistance Genes		
Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	None
Neomycin phosphotransferase II conferring resistance to kanamycin	*Tn5 E. coli* K12	None

*Determined using the amino acid sequence of the integrated gene and version 19 of the AllergenOnline Database maintained by the University of Nebraska – Lincoln; identity matches greater than 50% indicate possible cross-reactivity; with known allergens and require further testing, such as serum IgE binding, basophil histamine release or in vivo challenge; "≤" denotes that more than one hit occurred during the alignment and that the percent identity of all hits were was not greater than the stated value.

G. REGULATORY APPROVALS AROUND THE WORLD

Lacto-*N*-tetraose is GRAS in the United States for use in cow's milk-based term infant formula at levels up to 0.8 g/L and selected conventional foods and beverages at levels ranging from 1 to 20 g/kg (GRN 833, 2019). A Novel Food application for LNT has also been submitted to the European Commission by Glycom A/S proposing the use of LNT in infant and follow-on formulas at 0.8 and 0.6 g/L, respectively, and in selected conventional foods up to 10 g/kg. Although the application is still in review, the European Food Safety Authority agreed that the NOAEL for Glycom's LNT is 4,000 mg/kg bw/day, which was the highest dose tested in the 90-day oral toxicity study conducted by Phipps et al. (2018), intake of LNT at the proposed use levels is unlikely to exceed the intake level of naturally occurring LNT in breastfed infants on a body weight basis, the intake of other carbohydrates structurally related to LNT is not a safety concern, and that LNT is safe for use in infant and follow-on formulas at 0.8 and 0.6 g/L, respectively, and in selected conventional foods up to 10 g/kg (EFSA Panel on Nutrition et al., 2019).

VII. SUPPORTING DATA AND INFORMATION

A. REFERENCES

All information included in the following list of references is generally available.

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B. EXPERT PANEL STATEMENT

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of Lacto-*N*-tetraose (LNT) in non-exempt term infant formula has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). The safety of the intake of LNT in non-exempt term infant formula has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food and is based on generally available and accepted information.

The use of LNT as an ingredient for the intended use in infant formula has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. The subject of this GRAS Notice is a spray-dried, powdered food ingredient that contains not less than 75% LNT dry weight.

    a. LNT is a neutral, non-fucosylated oligosaccharide in human milk.

    b. The LNT that is the subject of this GRAS Notice is structurally identical to the LNT present in human breast milk.

    c. The subject of this GRAS Notice is manufactured by Jennewein in Food Safety System Certification (FSSC) 22000-, ISO 9001:2015-, GMP-, and/or International Featured Standards Food 6.1-compliant facilities. Jennewein is a Food Facility registered with FDA.

    d. The subject of this GRAS Notice is manufactured using a genetically engineered strain of *Escherichia coli* BL21(DE3). Because this organism does not possess the components required for *E. coli* pathogenicity, *E. coli* BL21(DE3) and strains derived from DE3 are non-pathogenic.

    e. All raw materials, processing aids, and food contact substances are GRAS and/or conform to the specifications stated in 21 CFR and/or the Food Chemicals Codex (FCC).

f. Fermentation by-products include lactose, the intermediate lactose-*N*-triose 2 (LNT II), and para-lacto-*N*-hexaose (pLNH), which are known human milk oligosaccharides; their presence in the finished ingredient is not of toxicological concern.

g. Product specifications are in place to control the levels of residual impurities and carbohydrate by-products, as well as heavy metals, microbes, and production organism-derived DNA and possible endotoxin, ensuring a consistent, safe, food-grade finished ingredient.

h. The available stability studies indicate a shelf-life of two years when stored from the date of production under ambient conditions.

2. Human milk oligosaccharides, including LNT, are resistant to the digestive enzymes in the gastrointestinal tract, poorly absorbed, and pass through the gastrointestinal tract where they are either fermented by the microbiota or excreted unchanged.

3. Published studies show that the amount of LNT in breast milk ranges from 0.003 to 6.7 g/L, with means and medians ranging from 0.1 to 3.9 and 0.2 to 2.1 g/L, respectively.

4. Genotoxicology and subchronic toxicology studies published by Phipps et al. (2018) show that LNT is not genotoxic and has a no observed adverse effect level (NOAEL) of 4 g/kg bw/day, which was the highest dose tested.

5. The addition of 1.82 g/L LNT in infant formula will result in mean and 90th percentile Estimated Daily Intakes (EDIs) of 2.52 and 4.95 g/day (0.4 and 0.7 g/kg bw/day), respectively, in infants 0 to 6 months-of-age, which represents the maximum level of intake because infants at this age consume infant formula as a sole source of nutrition.

6. The safety of exposure to Jennewein's LNT at its intended use level is supported by:

a. Published studies that quantitate the levels of LNT in human milk;

b. Analytical data demonstrating that the LNT produced by Jennewein is structurally identical to LNT from human milk;

c. Data demonstrating the qualitative and quantitative similarities between the subject of this GRAS Notice and the LNT ingredient tested by Phipps et al. (2018), which is the subject of GRN 833;

d. Corroborative genotoxicology and 90-day subchronic dietary toxicology studies conducted with a mixture of human milk oligosaccharides published by Parschat et al. (2020), which contained 26% (dry weight) of Jennewein's LNT).

e. A corroborative unpublished tolerance study in neonatal piglets conducted with a mixture of HMOs containing up to 1.6 g/L of Jennewein's LNT ingredient that showed an HMO mixture containing LNT was well-tolerated and supported normal growth in neonatal piglets.

Therefore, LNT is safe and GRAS at the proposed level of addition to the intended infant formula. Lacto-*N*-tetraose is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

Roger Clemens, DrPH, CNS, FACN, FIFT
GRAS Expert Panel Member
School of Pharmacy
University of Southern California

Signature: [redacted]

Date: March 19, 2020

A. Wallace Hayes, PhD, DABT, FATS, ERT
GRAS Expert Panel Member
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Date: March 19, 2020

Thomas E. Sox, PhD, JD
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Claire Kruger, PhD, DABT
Scientific Advisor to the Panel

Signature: [redacted]

Date: March 19, 2020

Spherix Consulting Group

October 19, 2020

Jason Downey, PhD
Regulatory Review Scientist
Division of Food Ingredients
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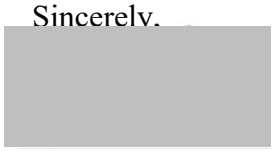
RE: Questions Regarding GRN 000923

Dear Dr. Downey:

In response to your email dated September 18, 2020, please find our responses to your request for additional information below. Importantly, although we believe that the published literature adequately supports an intended use level of 1.82 g/L in infant formula, Jennewein Biotechnology has decided to reduce the intended use level in this Notice to 0.8 g/L in non-exempt, cow's milk-based infant formula for term infants to be consistent with the intended use level established in GRN 000833. We hope that our responses adequately address your requests for additional information. If you have any additional questions or require any additional clarifications, please do not hesitate to contact me at dconze@spherixgroup.com.

Sincerely,



Dietrich B. Conze, Ph.D.
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FDA's questions regarding GRN 000923 are in italicized text and our responses are in plain text.

1. *On page 21, Jennewein states:*

   "Although this level is approximately 2.25-fold greater than what is GRAS (GRN 833, 2019), the results reported in the studies that quantitated the levels of LNT in breast milk indicate that the increased level of use will adequately accommodate variations in LNT levels due to ethnicity, Secretor and Lewis-blood group status, lactation period, and term vs. preterm birth."

   However, it is not clear from the notice on what basis Jennewein concluded that the higher use level of 1.82 g/L rather than 0.8 g/L is generally recognized as safe.

   OFAS notes the following:

   I. *Permitted maximum use level in the EU for LNT in infant formula is 0.8 g/L*[1].

   II. *The notifier for the previous GRN on LNT (GRN 833) concluded that a use level up to 0.8 g/L is GRAS based on datasets with large variations in the levels of LNT in breast milk. However, the notifier for GRN 833 reported an average of mean values of LNT in breast milk from studies stratified according to period of lactation: "colostrum," "transitional milk," "days 10-60 mature milk," and "after 2 months mature milk." This analysis demonstrated that the use level of 0.8 g/L was equal to or below the mean for three types of milk and within the mean and reported error for the fourth type of milk. We also note that Thurl et al., 2017*[2] *performed a systematic review and meta-analysis of the levels of human milk oligosaccharides in breast milk from secretor mothers and calculated the mean level of LNT to be 0.79 g/L with 95% confidence limits of 0.59 g/L and 0.98 g/L for term infants. Therefore, the proposed use level of 1.82 g/L in the current notice is over two times higher than the mean values estimated for LNT in breast milk by two different methodologies. It is unclear how Jennewein concluded that datasets generated since GRN 833 (2019) supersede the datasets that underpin the conclusions in GRN 833 and Thurl et al., 2017, to reflect more accurately the levels of LNT in human milk for infants 0-6 months.*

   III. *In Table 7 of the notice, Jennewein lists the mean LNT concentration from Azad et al. (2018) as 1.5 ± 0.7 g/L. However, in Supplemental Table 2 from the same publication, the mean LNT levels for all subjects is shown as 1480 ± 677* ***nmol/mL****. Assuming the molecular weight of LNT to be 707.6 g/mol, the mean LNT level expressed as* ***g/L*** *would be 1.047 g/L, a level much closer to the intended use level of 0.8 g/L in GRN 833 than Jennewein's proposed use level of 1.82 g/L.*

   IV. *We found no relevant published literature indicating that the maximum use level of 0.8 g/L is inappropriate nor literature providing compelling reasons or rationale as to why 1.82 g/L is more appropriate than the current use level for term infant formulae.*

On page 92 of the notice, Jennewein states: "The safety of exposure to Jennewein's LNT at its intended use level is supported by ... [p] published studies that quantitate the levels of LNT in human milk ..." Please provide a clear rationale, supported by publicly available data and information, that demonstrates Jennewein's LNT at the proposed higher use level is generally recognized and accepted by the scientific community to be GRAS for addition to nonexempt term infant formulae.

Thank you for alerting us to our calculation error regarding the LNT levels reported by Azad et al. (2018). The reported range, mean, and median reported for this study in Table 7 of GRN 000923 should be 0.07 – 3.9 g/L, 1.0 ± 0.48 g/L, and 0.9 g/L, respectively. Additionally, during a re-review of the Notice, we found that the highest mean stated in the sentence that summarizes the reported range, means and medians of LNT concentrations in breast milk from the published studies included in Table 7 is wrong (end of the 2nd paragraph in Section B. History of Exposure; page 16). The sentence should read, "Although the levels of LNT in human milk vary with ethnicity, Secretor and Lewis-blood group status, lactation period, and term vs preterm birth, the available studies show that the concentration of LNT in breast milk generally ranges from 0.003 to 8.5 g/L, with means and medians ranging from 0.1 to 3.9 and 0.2 to 2.1 g/L, respectively." Lastly, due to the precedent set in GRN 000833, Jennewein will reduce the use level to 0.8 g/L in non-exempt, cow's milk-based infant formula to be consistent with the use level that is currently GRAS. Thus, the use of LNT at the new intended use level is generally recognized and accepted by the scientific community to be GRAS for addition to non-exempt infant formula. To accommodate this change, please replace the text in Chapter III, Section C. Intended Uses and the last sentence of the 3rd paragraph in first section of Chapter VI, Narrative on the Conclusion of GRAS Status with the following, respectively:

"Jennewein intends to use LNT powder as a substitute for other forms of LNT in non-exempt, cow's milk-based infant formula for term infants at a level of 0.8 g/L, which is the same use level as what has been determined GRAS (GRN 000833, 2019)."

"Thus, Jennewein's intended use of 0.8 g LNT/L infant formula is well within the established range of LNT that occurs naturally in breast milk."

Also, as discussed in the Notice, only a small amount of human milk oligosaccharides (HMOs), such as LNT, are absorbed. The remaining fraction passes through the gastrointestinal tract where they are either fermented by microbiota or excreted unchanged in the feces. Synthetic and plant-based oligosaccharides, such as galacto-oligosaccharides (GOS), oligofructose, and fructo-oligosaccharides (FOS), which are not components of human milk, mimic the prebiotic effects of HMOs and have been determined safe for use in non-exempt infant formula at levels greater than the use of LNT (GRN 000392; GRN 000576; GRN 000797). Thus, in addition to the data summarized in GRN 000923, the safe use of LNT in infant formula is indirectly supported by a wealth of publicly available data demonstrating that the ingestion of other non-human oligosaccharides by infants is safe.

The pivotal information that supports the safe use of LNT in infant formula per the intend uses and use levels is appropriate manufacturing controls, food grade specifications, published, well-controlled sub-chronic rodent toxicology studies that confirm a lack of toxicity at relevant levels of exposure, and a neonatal piglet study that is the surrogate for tolerance and safety in infants. The corroborative data that supports the safe use of LNT in infant formula at the intended use level is that the intended use level falls within the established range for natural LNT concentrations in breast milk and adverse events associated with the use level have not been reported in the publicly available literature.

During our review of the publicly available literature, we found that non-secretor mothers produce LNT at levels higher than secretor mothers and the means reported in GRN 000833 and determined by Thurl et al. (2017)(Austin et al., 2019; Kunz et al., 2017; McJarrow et al., 2019; McGuire et al., 2017; Thurl et al., 2010; Gabrielli et al., 2011; Smilowitz et al., 2013; Van Niekerk et al., 2014; Hong et al., 2014; Sprenger et al., 2017). Thus, the means presented in GRN 000833 and calculated by Thurl et al. (2017) do not represent a comprehensive understanding of the naturally occurring levels of LNT in breast milk. To understand the relationship between the intended use level of 0.8 g/L, which was determined GRAS in GRN 833 and the levels of naturally occurring LNT in breast milk, the mean, median, central 68% confidence interval, 90th percentile, and distribution of the means reported in published studies were calculated in a non-parametric analysis using the following:

1. Means or medians reported in the published studies included in Table 7;

2. Means reported in the published studies included in the systematic review conducted by Thurl et al. (2017);

3. Means or medians reported in other published studies (Austin et al., 2019; Alderete et al., 2015; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Williams et al., 2017).

4. Means or medians reported in selected published studies excluded from the systematic review conducted by Thurl et al. (2017) (Bode et al., 2012; Kuhn et al., 2015; Newburg et al., 2004; Alderete et al., 2015; Austin et al., 2016; Kunz et al., 2017; Chaturvedi et al., 1997; Leo et al., 2009; Marx et al., 2014; Sumiyoshi et al., 2003).

Note that the selected studies excluded from the Thurl et al. (2017) systematic review were included in the analysis because LNT is higher in the breast milk of secretor mothers and excluding studies on the basis of "lactation period was not fitting" would artificially skew the data. Additionally, if only medians were reported in a study, then the medians were used in the analysis. The remaining studies excluded by Thurl et al. (2017) were not included in the analysis because they either did not quantify LNT in breast milk or provide the units of LNT that was quantified (Hunt et al., 2012; Galeotti et al., 2012; Galeotti et al., 2014).

A total of two hundred and twenty-four means and medians were extracted from the included studies and the resulting mean, median, upper and lower limits of the central 68% confidence interval, and 90th percentile were calculated to be 1.15, 0.97, 1.69, 0.54, 2.09 g/L, respectively. The distribution of the means is presented in Figure 1. Thus, the intended use level of 0.8 g/L falls below the mean of the means reported in the published studies included in the analysis.

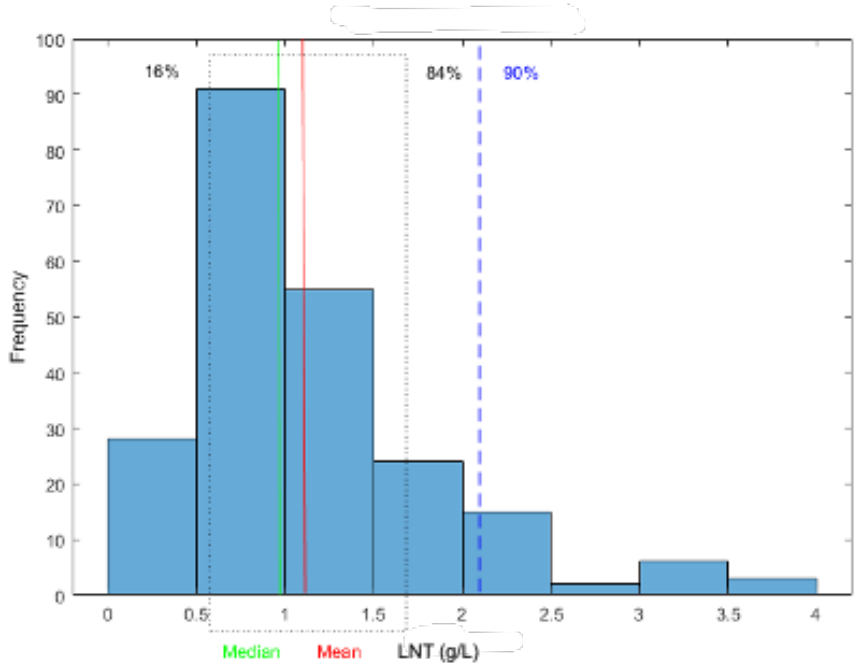

Figure 1. *The distribution, mean, median, the central 68% confidence interval, and 90th percentile of the mean/median levels of LNT in human milk. Two hundred and twenty-four means and medians were abstracted from the studies included in Table 7 of GRN 000923, reviewed by Thurl et al. (2017), as well as other publicly available studies. The histogram depicts the distribution of the LNT means/medians and the corresponding location of the mean (red line), median (green line), the upper and lower limits of the central 68% confidence interval (box with black dotted line), and the 90th percentile (blue dashed line).*

2. *On page 5, Jennewein indicates that the structure of their LNT is confirmed using LC-MS/MS and NMR spectroscopy. Because peak area was used to determine the amount of LNT, carbohydrates, lactose, lacto-N-triose II, para-lacto-N-hexose and glucose/galactose in the LNT ingredient, please provide a sample chromatogram for your LNT product with the various substances identified accordingly.*

As stated correctly in the question, the structure of Jennewein's LNT is confirmed using LC-MS/MS and NMR. However, for release of each batch of product, LNT content, as well as carbohydrate by-product content are determined using HPAEC-PAD (high pH anion exchange chromatography with pulsed amperometric detection). During this analysis, LNT is quantified using a calibration curve generated with an LNT standard of

known purity and the amount is presented as percent dry weight (% DW). As specified in Table 3 of GRN000923, only the carbohydrate by-product content is presented as percent area because for most of these substances, suitable standards with known purity are not available.

The representative chromatogram below shows HPAEC-PAD analysis of batch no. 16125019 (Figure 2). Lacto-N-tetraose is the main peak, eluting at approximately 10 min. The other peaks are the carbohydrate by-products, which include N-acetylglucosamine (GlcNAc), lactose, lacto-N-triose 2 (LNT2), lacto-N-biose (LNB), glucosyllactose (Glc-Lac), LNT-$(LNB)_1$, also known as para-lacto-N-hexaose (pLNH), LNT-GlcNAc, and LNT-$(LNB)_2$, also known as para-lacto-N-octaose (pLNO).

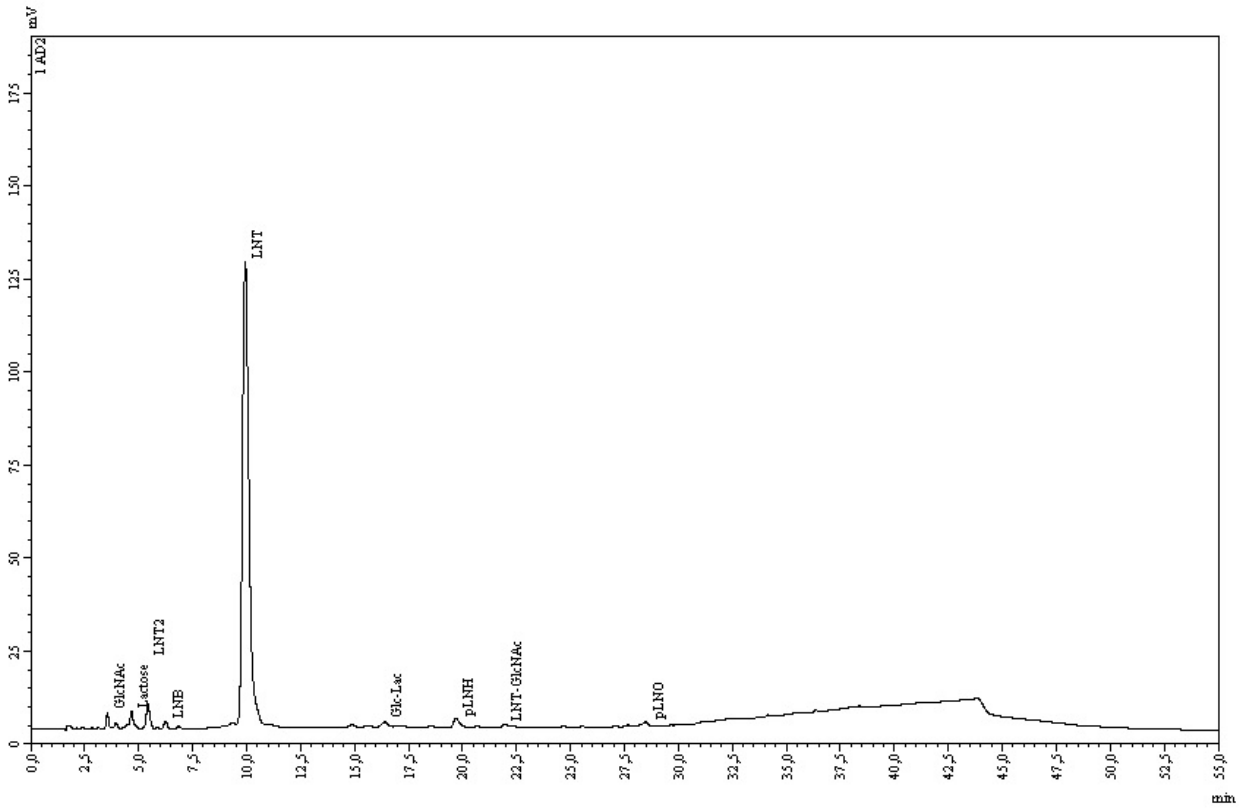

Figure 2. *Representative chromatogram of the high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis (Batch No. 16125019). LNT and carbohydrate by-product peaks have been labelled for clarity. Baseline drift is arising from elution gradient. Abbreviations: GlcNAc, N-Acetylglucosamine; LNT2, Lacto-N-Triose 2; LNB, Lacto-N-Biose; LNT, Lacto-N-Tetraose; Glc-Lac,Glucosyllactose; pLNH, para-Lacto-N-Hexaose, also known as LNT-$(LNB)_1$; LNT-GlcNAc; and pLNO, para-Lacto-N-Octaose, also known a LNT-$(LNB)_2$.*

3. Jennewein lists in Table 3 the results of the batch analysis as "ND" for most of the heavy metals. Please confirm that "ND" in Table 3 indicates that the heavy metal in the respective sample is not detected at the limit of detection of the analytical method. If our understanding is not correct, please indicate what is meant by "ND" in Table 3.

ND is an abbreviation for "not detected", which was mistakenly left out of the footer of Table 3. Therefore, for the heavy metal data in Table 3 where "ND" is stated, the heavy metal was not detected in the respective batch.

4. Jennewein indicates in Table 3 a specification of ≤ 25% for the sum of other carbohydrates. Please specify what are the "other carbohydrates" that could comprise up to 25% of LNT.

The "other carbohydrates" that could comprise up to 25% of LNT include:

1. N-acetylglucosamine (GlcNAc);
2. Lacto-N-biose (LNB);
3. Galactosyllactose;
4. Glucosyllactose;
5. LNT-$(LNB)_{n,}$ which includes LNT-$(LNB)_{2,}$ also known as para-lacto-N-octaose (pLNO);
6. LNT-$(LNB)_n$-N-GlcNAc, which includes LNT-$(LNB)_0$, also known as LNT-GlcNAc;
7. Total unspecific impurities.

These impurities and their relative amounts are listed in Table 8 of the Notice. Note that the subscript "n" in LNT-$(LNB)_n$ and LNT-$(LNB)_n$-GlcNAc denotes the number of repeating LNB units attached to LNT and can be any number starting from zero, with LNT-$(LNB)_0$-GlcNAc being LNT-GlcNAc. Additionally, the LNT-$(LNB)_n$-GlcNAc species included in "other carbohydrates" do not include para-lacto-N-hexaose because this by-product is specified with a limit of not more that 5 % (% area)(See Table 3 of the Notice).

5. Please indicate that all analytical methods are validated for their intended use.

All analytical methods are validated for their intended use.

6. *On page 9, Jennewein indicates that additional processing aids used in the manufacturing process comply with European Pharmacopoeia, United States Pharmacopeia-National Formulary, or Japanese Pharmacopoeia specifications or appropriate product monographs. Please confirm that all processing aids are approved for their respective use via a regulation in Part 21 of the U.S. Code of Federal Regulations (CFR), are the subject of an effective food contact notification, or are GRAS for that use in the U.S.*

   The materials used in the production of the LNT that is the subject of this Notice include the production strain, medium components, and physical processing aids. The physical processing aids are used in the downstream processing where LNT is purified from the fermentation medium and concentrated, and include activated carbon, ion exchange resins, and ultra- and nano-filtration membranes. All of these physical processing aids comply with conditions of use specified in the U.S. Code of Federal Regulations. The medium components are essential for the growth of the production strain and LNT production. Importantly, they are actively consumed by the production strain, which is removed prior to the downstream processing, considered to be "processing aids of a processing aid", and are not direct food additives. Additionally, if residues of these components remain in the LNT-containing medium after fermentation, they are removed from the product using ion exchange chromatography and electrodialysis. Because similar medium components are used in the production of other human oligosaccharides that are GRAS and received "no questions" letters from the Agency (GRN 000571, GRN 000650, GRN 000735, GRN 000833, GRN 000852), all of the medium components used in the production of the LNT that is the subject of this Notice are considered GRAS for their use.

7. *On page 9, Jennewein incorporates by reference the production process for LNT in GRN 571. We note that the fermentation medium described in GRN 571, in its Appendix J, includes added cobalt (II) chloride. In Table 4 of the present notice, Jennewein provides batch analyses for cobalt in LNT indicating that cobalt was present at levels above the limit of quantitation in three of the six batches. Under 21 CFR 189.120, food containing any added cobaltous salts, including cobalt (II) chloride, is deemed adulterated. Please describe how Jennewein will produce LNT in compliance with 21 CFR 189.120 and provide analytical data from five nonconsecutive batches of LNT that demonstrate LNT can be produced without added cobalt remaining in the product.*

   During downstream processing, the LNT-containing culture medium, which contains residual amounts of the salts required for the growth of the production strain JBT-LNT during fermentation, is subjected to ion exchange chromatography and electrodialysis to remove the elements from the finished product. To confirm that these processes remove cobalt, Jennewein quantitated the levels of cobalt in five batches of the finished ingredient (Table 2). Cobalt was below the limit of quantitation in all batches, indicating that the manufacturing process removed this medium element from the finished ingredient. Importantly, because of the regulatory status of cobalt (II) chloride, Jennewein will no longer use cobalt in their fermentation medium.

Table 1. Cobalt Analysis of Lacto-N-tetraose							
			Batch Number				
Element[1]	Method	LOQ	11022049	11023019	11023039	16125029	26125020
Cobalt (mg/kg)	PV-347 (ICP-MS)	0.04 mg/kg	<0.04	<0.04	<0.04	<0.04	<0.04
Abbreviations: ICP-MS, inductively coupled plasma mass spectrometry; LOQ, limit of quantitation [1]Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory.							

8. *Jennewein estimates exposure to LNT by multiplying the exposure in GRN 833 by the increase in use level for LNT in the current notice. We note that the intended uses in GRN 833 were in food categories in addition to infant formula. Therefore, this is not an appropriate method to estimate exposure for the use of LNT in the current notice. The additional food uses considered in GRN 833 could result in an exposure that is lower than that from the use in infant formula alone. Please provide an exposure estimate for the proposed use of LNT only in infant formula for infants aged 0-6 months and 7-12 months. Please also address a cumulative exposure to LNT from all uses.*

The subject of GRN 000923 is intended to be used in infant formula only, as a substitute for other LNT products that are currently GRAS for use in infant formula. Therefore, a calculation of the cumulative exposure to LNT is not needed. Additionally, because Jennewein will reduce the intended use level from 1.82 to 0.8 g/L (as discussed in our response to Question 1), the text in Chapter III, Section D. Estimated Daily Intake should read:

"To estimate exposure to the LNT that is the subject of this Notice, mean and 90th percentile energy intakes for infant boy and girls ages birth to approximately 6 months were determined by dividing their two-week caloric intake/kg body weight (bw) by the typical caloric of infant formula (670 g/L), resulting in the total infant formula intake/kg bw/day (Table 2; Martinez and Ballew, 2011; Fomon et al., 1993). The quotient was then multiplied by the intended use level of 0.8 g LNT/L, yielding the intake of LNT from infant formula/kg bw. The resulting mean and 90th percentile estimated LNT intakes for boys and girls combined from birth to 6 months-old ranged from 0.11 to 0.13 g/kg bw/day and 0.13 to 0.17 g/kg bw/day, respectively, and as the boys and girls aged, the mean and 90th percentile LNTs intakes decreased from approximately 0.14 and 0.17 g/kg bw/day to 0.11 and 0.13 g/kg bw/day, respectively (Table 2). Importantly, these estimates assume that infants will consume the LNT-containing infant formula as the sole source of nutrition. According to Grimes et al. (2015), who determined the dietary sources of total energy intake in infants and toddlers in the United States using the National Health and Nutrition Examination Survey 2005-2012 database, the actual total daily caloric intake from formula in infants 0 to 5.9 and 6 to 11.9 months-old is 65 and 47%, respectively. Thus, these assumptions and data indicate that LNT exposure estimates for infants ages birth to 5.9 months-old represent the most extreme and most conservative scenario and infants from 6 to 11.9 months old will consume less LNT than infants from birth to 5.9 months-old. Additionally, similar calculations have been used to support the safe use of other non-digestible carbohydrates such as short-chain fructooligosaccharides in infant

formula (GRN 537) and the caloric intakes used in the calculations are corroborated by caloric intake data from the Feeding Infants and Toddlers Study 2016 (FITS 2016), assuming a mean body weight of 5.4 kg (Bailey et al., 2018)."

Table 2. Estimated Daily Intake of LNT from Infant Formula

	Mean			90th percentile		
Interval (days)	kcal/kg/d[a]	Infant formula intake (L/kg)[b]	LNT intake (g/kg/day)	kcal/kg/d	Infant formula intake (L/kg)	LNT intake (g/kg/day)
Boys						
0-13	112.1	0.17	0.13	136.7	0.20	0.16
14-27	120.2	0.18	0.14	141.3	0.21	0.17
28-41	117.4	0.18	0.14	136.9	0.20	0.16
42-55	109.2	0.16	0.13	129	0.19	0.15
56-83	100.2	0.15	0.12	115.6	0.17	0.14
84-111	94.6	0.14	0.11	106.1	0.16	0.13
112-139	93.8	0.14	0.11	112.1	0.17	0.13
140-167	94.9	0.14	0.11	113.1	0.17	0.14
168-195	91.1	0.14	0.11	108.5	0.16	0.13
Girls						
0-13	110.7	0.17	0.13	135.5	0.20	0.16
14-27	117.6	0.18	0.14	138.9	0.21	0.17
28-41	114.2	0.17	0.14	136.8	0.20	0.16
42-55	108.2	0.16	0.13	127.4	0.19	0.15
56-83	100.2	0.15	0.12	114.4	0.17	0.14
84-111	95.2	0.14	0.11	106.8	0.16	0.13
112-139	97.5	0.15	0.12	113.1	0.17	0.14
140-167	93.6	0.14	0.11	113.3	0.17	0.14
168-195	90	0.13	0.11	107.9	0.16	0.13

[a]Obtained from Fomon, 1993.
[b]Calculated assuming that infant formula contains approximately 670 cal/L.

9. *Please state whether Escherichia coli BL21(DE3) strain "JBT-LNT" has been deposited in a recognized culture collection and provide the non-trade name designation. If the strain is not deposited, describe how the source was verified and identified.*

The strain has been deposited at DSMZ - German Collection of Microorganisms and Cell Cultures GmbH with the deposition number DSM 33494. The host strain from which JBT-LNT was generated was purchased from a commercial source with the genotype F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3). The identity of the genetically modified strain has been verified by its susceptibility and resistance to antibiotics, the presence of the genes that have been inserted via polymerase chain reaction, and its ability to produce LNT.

10. *Please state whether E. coli BL21(DE3) strain "JBT-LNT" is non-toxigenic.*

JBT-LNT is non-toxigenic.

11. Jennewein states that E. coli BL21(DE3) has an absence of genes encoding invasion factors, adhesion molecules, and enterotoxins associated with virulence; please state whether E. coli BL21(DE3) strain "JBT-LNT" has the same virulence profile.

Because Jennewein engineered JBT-LNT with genes with known functions that do not confer virulence using site-specific homologous recombination or transposition, JBT-LNT has the same virulence profile as *E. coli* BL21(DE3).

12. Please state whether E. coli BL21(DE3) strain "JBT-LNT" is capable of DNA transfer to other organisms.

JBT-LNT is not capable of DNA transfer to other organisms because *E. coli* BL21(DE3) is not able to transfer DNA to other organisms and Jennewein inserted only genetic elements that do not confer the ability to transfer DNA to other organisms using site-specific homologous recombination or transposition during the engineering of *E. coli* BL21(DE3) strain JBT-LNT.

13. Please state whether the fermentation process is conducted in a contained, sterile environment.

Jennewein's fermentation process is conducted in a contained, sterile environment.

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From: Dietrich Conze
To: Downey, Jason
Cc: Claire Kruger; Kathy Brailer
Subject: Re: GRN 923 - Request for Clarification
Date: Tuesday, November 3, 2020 11:42:06 AM

Hi Jason,

Your understanding is correct. Because the use of Jennewein's LNT in infant formula will be substitutional for other sources of LNT, there will be no increase in the overall exposure to LNT. Therefore, a calculation of the cumulative exposure to LNT is not needed.

Regards.
Dietz

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> On Nov 3, 2020, at 8:02 AM, Downey, Jason <Jason.Downey@fda.hhs.gov> wrote:
>
> **RE: GRN 923 – Jennewein Biotechnologie GmbH's LNT for use in non-exempt formula for term infants**
>
> Hi Dietz,
> While reviewing the October 19, 2020 amendment to GRN 923, which you submitted on behalf of Jennewein, we identified a point that needed clarification:
>
> - In response to our Question #8 from September 18, 2020, Jennewein indicates that their LNT would substitute for other LNT products that are currently GRAS for use in infant formula and therefore, a cumulative exposure is not needed. We would like Jennewein to clarify that they mean a cumulative exposure is not needed because their use would substitute for other uses of LNT and therefore, there would be no increase in the overall exposure to LNT. Please confirm that our understanding of this statement is correct.
>
> Please provide a response to this request as soon as you can but within 10 business days. If you are unable to complete the response within that time frame, please contact me to discuss further options. If you have questions or need further clarification, please feel free to contact me.
> Thank you,

From: Dietrich Conze
To: Downey, Jason
Cc: Claire Kruger; Kathy Brailer
Subject: Re: Notice of 90-day Extension - GRN 923
Date: Thursday, November 5, 2020 4:21:48 PM

Hi Jason,
Our client has alerted us to an error in method number used to enumerate *Enterobacteriaceae* (see Table 3 in Notice). The method number listed for *Enterobacteriaceae* is ISO 21528-1, which is wrong. The correct method number is ISO 21528-2.

Regards and I apologize for the confusion.
Dietz

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> On Nov 5, 2020, at 1:35 PM, Downey, Jason <Jason.Downey@fda.hhs.gov> wrote:
>
> **RE: GRN 923 (Jennewein's LNT)**
> Hi Dietz,
> This email is to inform you that, in accordance with 21 CFR 170.265 (b)(2), FDA is extending the normal 180-day review timeframe by 90 days. The original 180-day date is November 10, 2020.
> Regards,
> Jason
>
> **Jason Downey, PhD**
> *Regulatory Review Scientist*
> *Division of Food Ingredients*
> **Center for Food Safety and Applied Nutrition**
> **Office of Food Additive Safety**
> **U.S. Food and Drug Administration**
> jason.downey@fda.hhs.gov
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> <image001.png>
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> <image002.jpg> <image003.jpg> <image004.jpg> <image005.jpg> <image006.jpg>

From: Dietrich Conze
To: Downey, Jason
Cc: Claire Kruger; Kathy Brailer
Subject: Re: Notice of 90-day Extension - GRN 923
Date: Tuesday, November 17, 2020 8:27:28 AM

Hi Jason,

Ellen Anderson, who is coordinating the review of another one of our HMO GRAS notices, alerted me to two errors in the estimated daily intake assessments (Table 2 in our October 19, 2020 amendment). The first error is that the first interval should be 8-13 days, not 0-13 days. The second error is that we used the 50th percentile energy intakes reported by Fomon (1993), not the means. After re-reviewing the energy intakes reported by Fomon (1993), it is apparent that 50th percentile intakes are not dramatically different from the means, differing by less than 1 kcal/g/d. Therefore, the estimated daily intakes calculated with the means would not be dramatically different that those calculated with the 50th percentiles. Please let me know if you would like me to provide you with a new Table 2 with the correct information.

Regards.
Dietz

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> On Nov 6, 2020, at 1:18 PM, Downey, Jason <Jason.Downey@fda.hhs.gov> wrote:
>
> Hi Dietz,
> I'm not aware of additional questions or clarification regarding GRN 923 at this time, but the notice is still under review. Should we identify any going forward, I'll be sure to reach out with those as quickly as I can.
> Jason
>
> ---
>
> **From:** Dietrich Conze <dconze@spherixgroup.com>
> **Sent:** Friday, November 6, 2020 1:13 PM
> **To:** Downey, Jason <Jason.Downey@fda.hhs.gov>
> **Cc:** Claire Kruger <ckruger@spherixgroup.com>; Kathy Brailer <kbrailer@spherixgroup.com>
> **Subject:** Re: Notice of 90-day Extension - GRN 923

From: Dietrich Conze
To: Downey, Jason
Cc: Claire Kruger; Kathy Brailer
Subject: Re: GRN 923 - Literature Search
Date: Thursday, January 21, 2021 4:20:12 PM

Hi Jason,
Happy New Year to you too! Yes, a literature search was conducted to include all relevant published safety data on the intended use of LNT, not just the clinical data. The literature search was based on the results of the literature search conducted in GRN 833, which documents the safe use of LNT in non-exempt infant formula and selected conventional foods, and then extended up to the Expert Panel meeting date of March 19, 2020 using Pubmed. Importantly, GRN 923 includes all relevant published safety data on the intended use of LNT in non-exempt term infant formula including the safety of the production organism, the absorption, distribution, metabolism, and excretion of HMOs, the toxicology studies conducted on LNT alone and mixtures containing LNT, an unpublished neonatal piglet study conducted on a mixture containing LNT, and the allergenicity of the LNT-containing product.

Dietz

> On Jan 21, 2021, at 9:35 AM, Downey, Jason <Jason.Downey@fda.hhs.gov> wrote:
>
> Hi Dietz and Belated Happy New Year,
> We are wrapping up our evaluation of GRN 923 (Jennewein's LNT). To finish, I need a quick clarification on one point. On the notice's page 76, Jennewein states, "Additionally, a search of PubMed on February 24, 2020 using the search terms "lacto-N-tetratose" and "clinical" yielded no new clinical studies." I could not find a reference to an updated literature search for the intended use of LNT in general. Please confirm that a literature search was conducted to include all relevant published safety data on the intended use of LNT, not just clinical data, and specify the databases that were searched and the date through which the search was conducted.
> Please let me know if you have any questions.
> Thank you!
> Jason
>
> **Jason Downey, PhD**
> *Regulatory Review Scientist*
>
> **Division of Food Ingredients**
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