Spherix Consulting Group

February 12, 2019

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 GRAS determination titled "Generally Recognized As Safe (GRAS) Notification for the Use of Lacto-N-neotetraose in Non-Exempt Term Infant Formula, Conventional Foods, and Beverages constitutes a new notification. Although lacto-N-neotetraose is GRAS, the subject of the enclosed Notice is produced by fermentation using two novel genetically engineered strains of *E. coli* BL21 (DE3) unlike the subjects of GRN 547 and 659.

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

Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310, Rockville, MD 20852
301-557-0375; info@spherixgroup.com

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
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):* 08/01/2019

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-neotetraose (LNnT)

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

1 paper copy and 1 CD containing all files

3. For paper submissions only:

Number of volumes 1

Total number of pages ~700

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 547

☐ 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 659

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 LNnT as an ingredient in non-exempt term infant formula, follow-on formula, and conventional foods.

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-neotetraose (LNnT)
(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: 2020.02.12 10:54:57 -05'00'	Dietrich B. Conze, PhD, Managing Partner	02/12/2020

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 LNnT GRAS Notification - Final 2-12-2020.pdf	Submission
	Alderete 2015.pdf	Submission
	Archer et al 2011 625.pdf	Submission
	Asakuma et al 2008.pdf	Submission
	Asakuma et al 2011.pdf	Submission
	Austin 2016.pdf	Submission
	Austin 2019.pdf	Submission
	Azad 2018.pdf	Submission
	Bao et al 2013.pdf	Submission

PART VIII – LIST OF ATTACHMENTS *(continued)*

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)
	Chart et al 2010.pdf	Submission
	Chaturvedi 1997.pdf	Submission
	Chaturvedi 2001.pdf	Submission
	Commission Implementing Reg 2018-1023.pdf	Submission
	Committee on Nutrition 2000.pdf	Submission
	Comstock 2017.pdf	Submission
	Coppa 2001.pdf	Submission
	Coulet 2013.pdf	Submission
	Daegelen et al 2009.pdf	Submission

PART VIII – LIST OF ATTACHMENTS *(continued)*

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Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Davies 1994.pdf	Submission
	Dotz 2014.pdf	Submission
	EFSA 2015.pdf	Submission
	Elison 2016.pdf	Submission
	Ellis et al 2001.pdf	Submission
	Engfer 2000.pdf	Submission
	Erney 2000.pdf	Submission
	Gabrielli 2011.pdf	Submission
	Galeotti 2012.pdf	Submission

PART VIII – LIST OF ATTACHMENTS *(continued)*

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Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Gnoth 2000.pdf	Submission
	Goehring 2014.pdf	Submission
	Gridneva 2019.pdf	Submission
	Harper et al 2011.pdf	Submission
	Hester et al 2012.pdf	Submission
	Hoess 1990.pdf	Submission
	Jeong et al 2009.pdf	Submission
	Jost 2015.pdf	Submission
	Kuntz 2019.pdf	Submission

PART VIII – LIST OF ATTACHMENTS *(continued)*

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Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Kunz 1999.pdf	Submission
	Lambert et al 2007.pdf	Submission
	Lampe et al 1999.pdf	Submission
	Larsson 2019.pdf	Submission
	Larsson 2019 - Corrigendum.pdf	Submission
	Leo et al 2010.pdf	Submission
	Milani et al 2017.pdf	Submission
	National Institutes of Health, 2019.pdf	Submission
	Nakhla et al 1999.pdf	Submission

PART VIII – LIST OF ATTACHMENTS *(continued)*

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Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Nowak-Wegrzyn 2019.pdf	Submission
	Obermeier 1999.pdf	Submission
	Prieto 2005.pdf	Submission
	Puccio 2017.pdf	Submission
	Rudloff 1996.pdf	Submission
	Rudloff 2006.pdf	Submission
	Rudloff 2012.pdf	Submission
	Rudloff and Kunz 2012.pdf	Submission
	Ruhaak 2014.pdf	Submission

PART VIII – LIST OF ATTACHMENTS *(continued)*

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)
	Santos-Fandila 2014.pdf	Submission
	Section on Breastfeeding 2012.pdf	Submission
	Smilowitz 2014.pdf	Submission
	Sprenger 2017.pdf	Submission
	Studier et al 2009.pdf	Submission
	Sumiyoshi et al 2003.pdf	Submission
	Thurl 1997.pdf	Submission
	Thurl et al 2010.pdf	Submission
	Vandenplas 2018.pdf	Submission

PART VIII – LIST OF ATTACHMENTS *(continued)*

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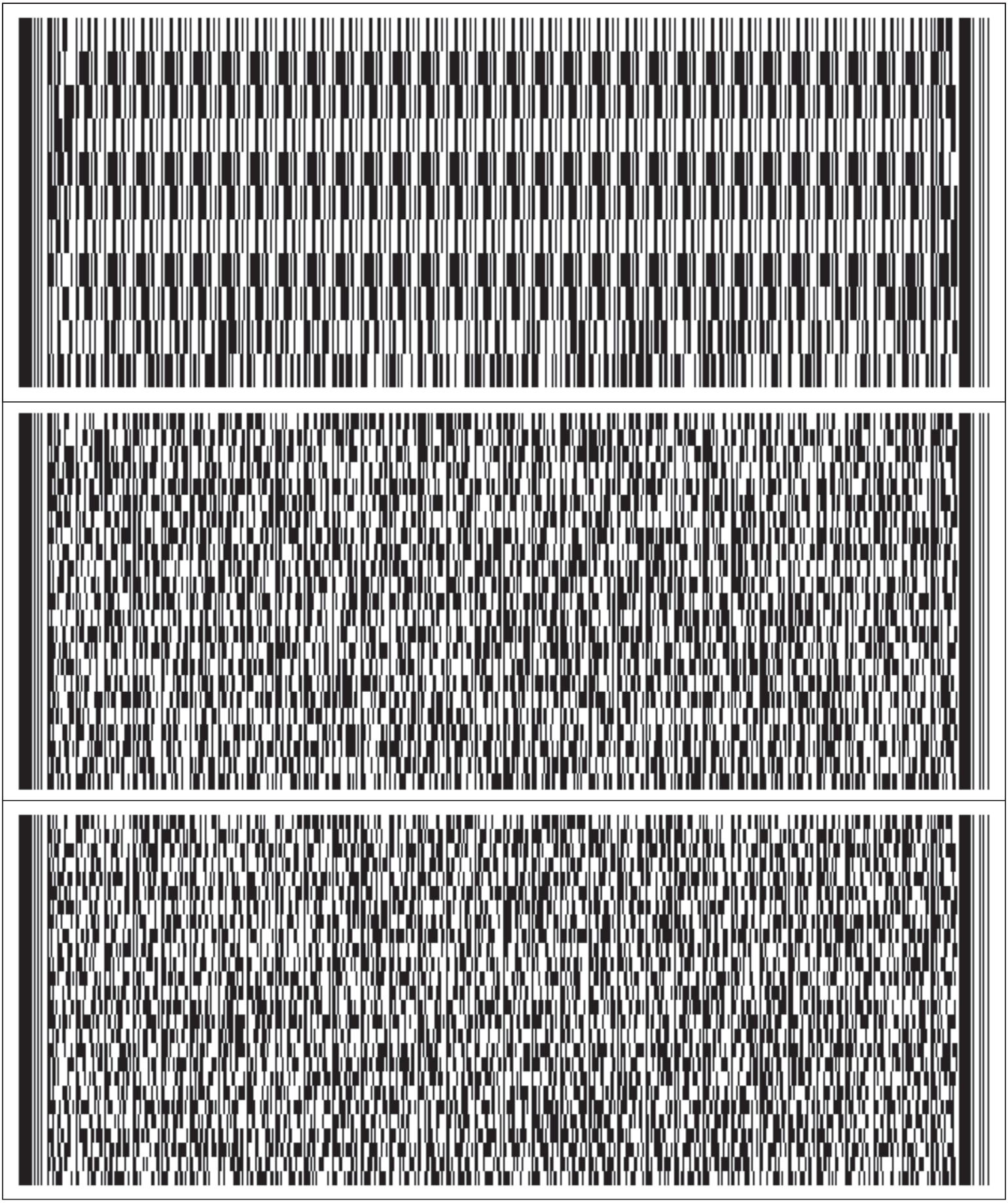
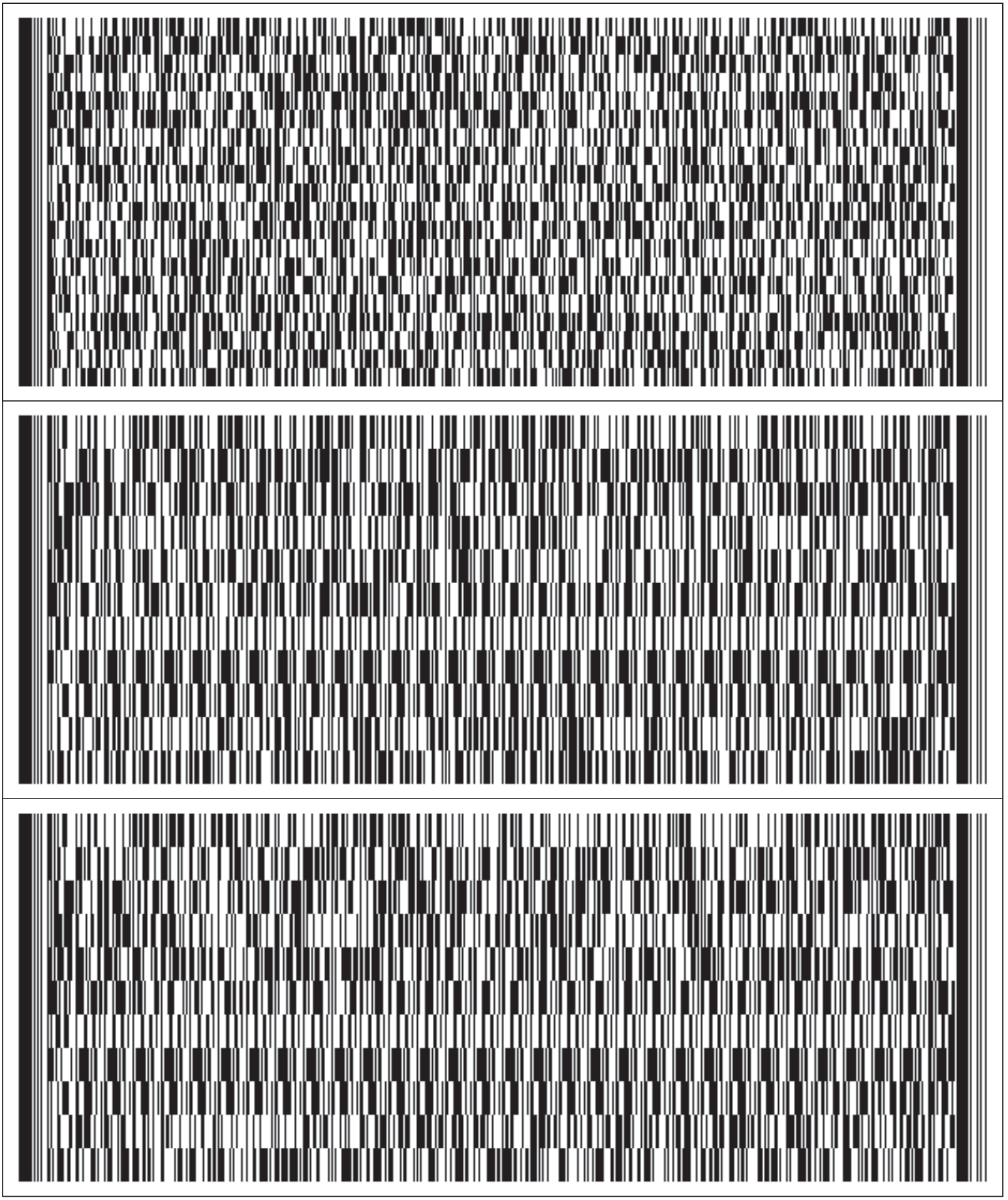
Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Van Niekerk 2014.pdf	Submission
	Vazquez 2017.pdf	Submission
	Williams 2017.pdf	Submission





GRAS Determination for the Use of Lacto-*N*-*neo*tetraose in Non-Exempt Term Infant Formula, Conventional Foods, and Beverages

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
USA

February 6, 2020

TABLE OF CONTENTS

I. SIGNED STATEMENT OF THE CONCLUSION OF GENERALLY RECOGNIZED AS SAFE (GRAS) AND CERTIFICATION OF CONFORMITY TO 21 CFR §170.205-170.260 1

A. SUBMISSION OF GRAS NOTICE 1

B. NAME AND ADDRESS OF THE SPONSOR 1

C. COMMON OR USUAL NAME 1

D. TRADE SECRET OR CONFIDENTIAL INFORMATION 1

E. INTENDED USE 1

F. BASIS FOR GRAS DETERMINATION 1

G. PREMARKET APPROVAL 3

H. AVAILABILITY OF INFORMATION 3

I. FREEDOM OF INFORMATION ACT (FOIA) 4

J. INFORMATION INCLUDED IN THE GRAS NOTIFICATION 4

II. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT OF THE NOTIFIED SUBSTANCE 5

A. COMMON OR USUAL NAME 5

B. CHEMICAL NAME 5

C. MOLECULAR FORMULA AND MASS 5

D. STRUCTURAL FORMULA 5

E. DESCRIPTION OF LACTO-N-NEOTETRAOSE 5

F. PRODUCTION PROCESS 6

1. Description of the Bacterial Strains Used for LNnT Production 6

2. Manufacturing 10

G. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES 13

1. Product Specifications 13

2. Other Quality Attributes 16

H. STABILITY OF LNnT POWDER 17

1. Stability of LNnT Powder 17

III. DIETARY EXPOSURE 18

A. INTENDED EFFECT 18

B. HISTORY OF EXPOSURE 18

C. INTENDED USES 18

D. ESTIMATED DAILY INTAKE 19
1. Infant Formula 20
2. Selected Conventional Foods and Beverages 20
IV. SELF-LIMITING LEVELS OF USE 21
V. COMMON USE IN FOOD BEFORE 1958 22
VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS 23
A. CHEMICAL EQUIVALENCE OF LNnT PRODUCTS 25
B. SAFETY OF THE PRODUCTION ORGANISMS 26
C. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION 27
D. TOXICOLOGY STUDIES 28
1. Genotoxicity Studies 29
2. Subchronic Toxicology Studies 30
E. CLINICAL STUDIES 32
F. OTHER CORROBORATIVE INFORMATION 34
G. ALLERGENICITY 34
H. REGULATORY APPROVALS AROUND THE WORLD 36
VII. SUPPORTING DATA AND INFORMATION 37
A. REFERENCES 37
B. EXPERT PANEL STATEMENT 47

LIST OF TABLES

Table 1. Genetic Manipulations in PS-LNnT-JBT 7
Table 2. Genetic Manipulations in DS-LNnT-JBT 9
Table 3. Lacto-N-neotetraose Product Specifications and Batch Data 14
Table 4. Elemental Analysis of the LNnT[1] 16
Table 5. Intended Uses of LNnT[1] 19
Table 6. Carbohydrate By-Product Profile of Lacto-N-neotetraose[1] 26
Table 7. Percent Identity of the Genetic Manipulations in Production Strains with Known Allergens 35

LIST OF FIGURES

Figure 1. Synthesis of LNnT with PS-LNnT-JBT 8
Figure 2. Degradation of Intermediates and By-products with DS-LNnT-JBT 10
Figure 3. Flow Diagram of LNnT Production 12

LIST OF ABBREVIATIONS

2'FL: 2'-fucosyllactose

araA: Arabinose isomerase

BW: Body weight

DBPCFC: Double-blind, placebo-controlled, food challenges

DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen

EDI: Estimated daily intake

EFSA: European Food Safety Authority

EU: Endotoxin unit

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

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

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

GOS: Galactooligosaccharides

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

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

HMO: Human milk oligosaccharide

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

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

LNT II: Lacto-N-triaose II

LOD: Limit of detection

LOQ: Limit of quantitation

ND: Not detected

NHANES: National Health and Nutrition Examination Surveys

NIH: National Institutes for 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

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*-*neo*tetraose (LNnT)

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 LNnT as an ingredient in non-exempt term infant formula, follow-on formula, and conventional foods.

F. BASIS FOR GRAS DETERMINATION

Lacto-*N*-*neo*tetraose for the intended uses 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 LNnT has been determined to be GRAS by demonstrating that the safety of this 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 proposed use of LNnT as an ingredient for the intended uses in infant formula, and conventional foods and beverages has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. LNnT is one of the most abundant (0.1-0.6%) oligosaccharides in human milk. Human milk oligosaccharides, including LNnT, 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.

2. The LNnT that is the subject of this GRAS determination is a spray-dried, powdered food ingredient that contains not less than 92% LNnT and is manufactured by fermentation using two genetically engineered strains of *Escherichia coli* BL21(DE3). By-products include lactose, the intermediate lactose-N-triaose 2 (LNT II), and *para*-lacto-*N*-*neo*hexaose (pLNnH), which are also human milk oligosaccharides and their presence in the finished ingredient is not unexpected.

3. The subject of this GRAS Notification is structurally identical to human milk LNnT and is chemically equivalent to the subjects of GRAS Notifications (GRNs) 547 and 659, both of which received "no questions" letters from FDA.

4. Jennewein is a Food Facility registered with FDA.

5. LNnT 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.

6. LNnT is manufactured with the help of the two genetically engineered production strains of *Escherichia coli* BL21(DE3). Because *E. coli* BL21(DE3) does not possess the components required for *E. coli* pathogenicity, the production strains are non-pathogenic.

7. All raw materials, processing aids, and food contact substances are GRAS.

8. 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 endotoxin, ensuring a consistent, food-grade finished ingredient.

9. Because the subject of this GRAS notification is structurally identical to the LNnT present in human breast milk and subjects of GRN 547 and 659, and chemically equivalent to the subjects of GRN 547 and 659, the genotoxicology and 90-day toxicology studies published by Coulet et al. (2013), which support the safe use of

the subject of GRN 547, are the pivotal studies that support the safe use of LNnT in infant formulas, selected conventional foods and beverages.

a. LNnT is not genotoxic.

b. The NOAEL for orally administered LNnT reported by Coulet et al. (2013) is at least 5000 mg/kg/day.

c. Additional genotoxicology and 90-day toxicology studies summarized in GRN 659 corroborate the results published by Coulet et al. (2013).

10. Clinical studies have reported that the ingestion of 1 g/L and 20 g/d of LNnT is well-tolerated in infants and adults, respectively.

11. The intended uses and corresponding use levels will result in a mean and 90th percentile estimated daily intakes (EDIs) of 510 and 730 mg/day (83.2 and 133.9 mg/kg bw/day, respectively) in 0 to 6-month old infants consuming LNnT-containing formulas, mean and 90th percentile EDIs of 420 and 660 mg/day in 7 to 12 month old infants, and mean and 90th percentile EDIs of 304 and 646 mg/day (8.1 and 16.8 mg/kg bw/day, respectively) from selected conventional foods and beverages.

12. The EDI is substantially below the safe level of intake for LNnT determined in the toxicology and corroborating clinical studies, thereby establishing the safety of LNnT ingestion from the intended uses and use levels.

Therefore, LNnT is safe and GRAS at the proposed levels of addition to the intended infant and follow-on formula, products for children, and conventional foods and beverages. Lacto-*N*-*neo*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, DABT, 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.

Signature of Authorized Representative of
Jennewein Biotechnologie GmbH

10/2/20
Date

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

A. COMMON OR USUAL NAME

Lacto-*N*-*neo*tetraose (LNnT; CAS No. 13007-32-4)

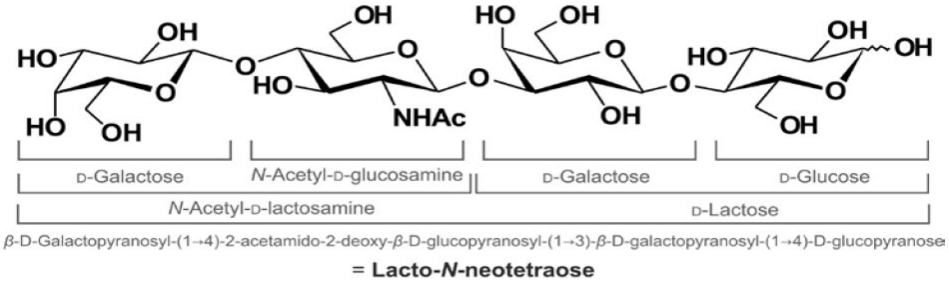
B. CHEMICAL NAME

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

C. MOLECULAR FORMULA AND MASS

$C_{26}H_{45}NO_{21}$; 707.63Da

D. STRUCTURAL FORMULA



E. DESCRIPTION OF LACTO-N-NEOTETRAOSE

Lacto-*N*-*neo*tetraose is one of the most abundant oligosaccharides in human milk. It is a tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose, and D-glucose (see Structural Formula). Synthetic forms of LNnT produced by chemical synthesis and fermentation are GRAS for use in infant formula and conventional foods (GRN 547; GRN 659). The LNnT that is the subject of this GRAS determination is a spray-dried, powdered product synthesized by fermentation from D-glucose, fructose, sucrose, or glycerol using two genetically engineered strains of *Escherichia coli* BL21(DE3). The finished product contains not less than 92% LNnT and is chemically equivalent to the subjects of GRN 547 and 659. Additionally, the LNnT produced by Jennewein is structurally identical to the LNnT found in human breast milk as confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, double-quantum filtered $^{1}H^{1}H$-COSY NMR spectroscopy, phase-sensitive $^{1}H^{13}C$-heteronuclear single quantum correlation (HSQC) NMR spectroscopy, phase-sensitive $^{1}H^{13}C$-heteronuclear multiple bond correlation (HMBC) NMR spectroscopy, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). By-products include lactose, the intermediate lactose-*N*-triaose (LNT II), and *para*-lacto-*N*-*neo*hexaose (pLNnH), which are not unexpected due to the specificity of the

enzymes used to synthesize LNnT. Importantly, these carbohydrate by-products are also known as human oligosaccharides and similar to those reported in GRN 659.

F. PRODUCTION PROCESS

Lacto-*N*-*neo*tetraose is manufactured from D-glucose, fructose, sucrose, or glycerol in a 2-step fed-batch fermentation process using two genetically engineered strains of *E. coli* BL21 (DE3), PS-L*N*nT-JBT and DS-L*N*nT-JBT. The first step is the synthesis of LNnT from lactose using PS-L*N*nT-JBT. The second step is hydrolysis of unreacted LNnT precursors remaining in the fermentation medium using DS-L*N*nT-JBT, which increases the purity and yield of the finished product. Following fermentation, the LNnT-containing medium is removed from the biomass and LNnT is concentrated and refined in a series of steps generating a white to off-white powder.

1. Description of the Bacterial Strains Used for LNnT Production

PS-L*N*nT-JBT and DS-L*N*nT-JBT were derived from the host strain *E. coli* BL21(DE3), purchased from Novagen (now part of Merck Millipore, Darmstadt, Germany). Both PS-L*N*nT-JBT and DS-L*N*nT-JBT are stored as glycerol stocks at -80°C in a master cell bank at the production sites and will be deposited at the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen)-German Collection of Microorganisms and Cell Cultures. The master cell banks of PS-L*N*nT-JBT and DS-L*N*nT-JBT are used to produce working cell banks, which are then used to directly inoculate fermentation pre-cultures.

a. Generation of PS-LNnT-JBT

To generate PS-L*N*nT-JBT, a variety of genes were either deleted, inactivated, or ectopically overexpressed to allow the bacterium to grow on sucrose, produce high levels of uridine diphosphate-galactose, and synthesize lactose and LNnT (Table 1; Figure 1). All heterologous genes to *E. coli* BL21(DE3) were purchased as synthetic DNA constructs containing the gene of interest and LoxP-flanked antibiotic resistance genes. The constructs were then amplified by polymerase chain reaction (PCR) and linked to LoxP-flanked antibiotic resistance genes. All homologous genes from *E. coli* BL21(DE3) and *E. coli* K12 were amplified from genomic DNA by PCR. All genomic deletions were performed by λ red recombinase-mediated homologous recombination (Datsenko and Wanner, 2000). All ectopically overexpressed genes were introduced by transposition (Lampe et al., 1999). Arabinose isomerase (araA) was inactivated by mutagenesis using mismatch oligonucleotides to prevent L-arabinose degradation (Ellis et al., 2001) and to allow for arabinose-induced expression of λ red recombinase and transposase required for transposition. The antibiotic resistance genes that were used for positive selection of homologous recombinants and integrants were excised from the

genome by Cre-mediated recombination (Lambert et al., 2007; Hoess and Abremski, 1990). All gene deletions and insertions were then verified by PCR using oligonucleotides specific to the coding sequence and PS-L*N*nT-JBT 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 growth at 42°C, ampicillin sensitivity, and failure to amplify plasmid specific DNA. Although PS-L*N*nT-JBT possesses the zeocin (*Sh ble*) and chloramphenicol (*cat*) resistance genes that were used for integrant selection, no plasmids or other episomal vectors remain in the genome, thus conferring a high degree of genetic stability.

Table 1. Genetic Manipulations in PS-LNnT-JBT

Gene	Function	Origin of the Gene	Manipulation	Effect
lacZ	β-galactosidase	*E. coli* BL21(DE3)	Deletion	To prevent hydrolysis of lactose
lacA	Galactosidase O-acetylglucosaminyltransferase	*E. coli* BL21(DE3)	Deletion	To prevent acetylation of lactose and subsequent carbohydrate metabolism
araA	Arabinose isomerase	*E. coli* BL21(DE3)	Inactivation	To prevent arabinose degradation
nagA	N-acetylglucosamine-6-phosphate deacetylase	*E. coli* BL21(DE3)	Deletion	To prevent N-acetyl-glucosamine catabolism
nagB	Glucosamine-6-phosphate deaminase	*E. coli* BL21(DE3)	Deletion	
wcaJ	UDP-glucose:undecaprenyl phosphate glucose-1 phosphate transferase	*E. coli* BL21(DE3)	Deletion	To prevent colonic acid synthesis
lacY	Lactose permease	*E. coli* K12	Overexpression	Facilitate lactose uptake
pgm	Phosphoglucomutase	*E. coli* K12	Overexpression	To enhance UDP-galactose synthesis
galE	UDP-galactose-4-epimerase	*E. coli* K12	Overexpression	
galU	UTP-glucose-1-phosphate uridyltransferase	*E. coli* K12	Overexpression	
galF	Regulatory subunit of GalU	*E. coli* K12	Overexpression	
lgtA	β-1,3-N-acetylglucosaminyltransferase	*Neisseria meningitidis*	Overexpression	To allow for the synthesis of LNT II
galT	β-1,4-galactosyltransferase	*Aggregatibacter aphrophilus*	Overexpression	To allow for the synthesis of lactose and LNT
Sh ble	Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	Overexpression	To allow for the selection of integrants during genetic engineering
cat	Chloramphenicol acetyl transferase conferring resistance to chloramphenicol	*Shigella sonnei*	Overexpression	

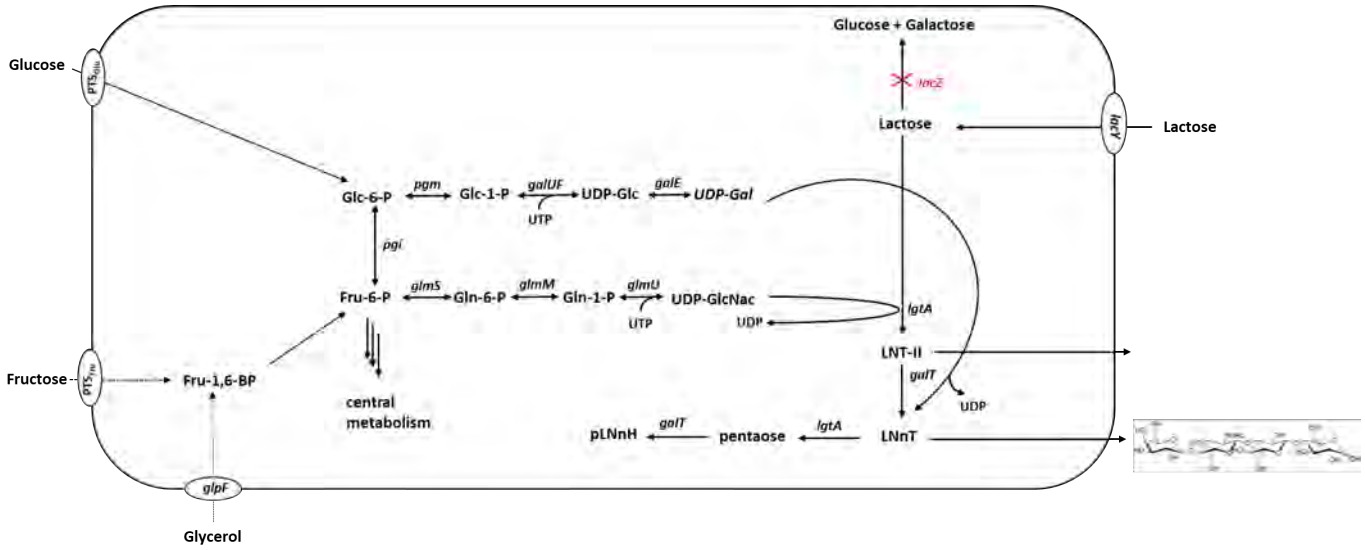


Figure 1. Synthesis of LNnT with PS-LNnT-JBT

Glc-6-P: glucose-6-phosphate, Glc-1-P: glucose-1-phosphate, UDP-Glc: UDP-glucose, UDP-Gal: UDP-galactose, Gln-6-P: glucosamine-6-phosphate, Gln-1-P: glucosamine-1-phosphate, UDP-GlcNAc: UDP-N-acetylglucosamine, Fru-6-P: fructose-6-phosphate, Fru-1,6-BP: Fructose-1,6-bisphosphate, LNT II: Lacto-N-triaose II, LNnT: Lacto-*N*-*neo*tetraose, pLNnH: *para*-Lacto-*N*-neohexaose.

b. Generation of DS-LNnT-JBT

To generate DS-L*N*nT-JBT, a variety of genes were ectopically overexpressed by transposition to allow the bacterium to hydrolyse lactose, cleave the *N*-acetylglucosamine from LNT II, and improve the degradation of the monosaccharide impurities *N*-acetylglucosamine and galactose (Table 2; Figure 2). All heterologous genes to *E. coli* BL21(DE3) were purchased as synthetic DNA constructs and amplified by PCR and flanked with antibiotic resistance genes. All homologous genes from *E. coli* BL21(DE3) and *E. coli* K12 were amplified from genomic DNA by PCR and flanked with antibiotic resistance genes. AraA was inactivated by mutagenesis using mismatch oligonucleotides to facilitate arabinose-induced transposase expression, and subsequent transposition. All gene inactivations and insertions were then verified by PCR using oligonucleotides specific to the coding sequence and DS-L*N*nT-JBT genomic DNA. Loss of the plasmid that encoded the transposase, which contained an ampicillin resistance gene and a temperature-sensitive origin of replication, was confirmed by growth at 42°C, ampicillin sensitivity, and failure to amplify plasmid specific DNA. Thus, although the DS-L*N*nT-JBT genome contains the streptomycin (*aad1*), zeocin (*Sh ble*), kanamycin (*nptII*), and gentamycin (*aacC1*) resistance genes used for integrant selection, all plasmids and other episomal vectors were removed from genome.

Table 2. Genetic Manipulations in DS-LNnT-JBT

Gene	Function	Origin of the gene	Manipulation	Function
araA	Arabinose isomerase	*E. coli* BL21(DE3)	Inactivation	To prevent arabinose degradation
lacZ	β-galactosidase	*E. coli* BL21(DE3)	Overexpression	To enhance lactose degradation
lacY	Lactose permease	*E. coli* K12	Overexpression	Facilitate lactose uptake
galE	UDP-galactose-4-epimerase	*E. coli* K12	Overexpression	To restore galactose metabolism
galT	Galactose-1-phosphate uridylyltransferase	*E. coli* K12	Overexpression	
galK	Galactokinase	*E. coli* K12	Overexpression	
galM	Galactose mutarotase	*E. coli* K12	Overexpression	
bbhI	β-*N*-acetylhexosaminidases	*Bifidobacterium bifidum*	Overexpression	To allow for the degradation of LNT II
nagA	N-acetylglucosamine-6-phosphate deacetylase	*E. coli* BL21(DE3)	Overexpression	To enhance the degradation of N-acetylglucosamine
nagB	Glucosamine-6-phosphate deaminase	*E. coli* BL21(DE3)	Overexpression	
nagE	PTS system N-acetylglucosamine-specific EIICBA component	*E. coli* BL21(DE3)	Overexpression	
Sh ble	Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	Overexpression	To allow for the selection of integrants during genetic engineering
aacC1	Gentamycin 3'-acetyltransferase conferring resistance to gentamycin	*Acinetobacter baumannii AYE*	Overexpression	
nptII	Neomycin-phosphotransferase II conferring resistance to kanamycin	TTn5 *E. coli* K12	Overexpression	
aad1	Aminoglycoside adenyltransferase conferring resistance to streptomycin	*Staphylococcus aureus* strain RIGLD7	Overexpression	

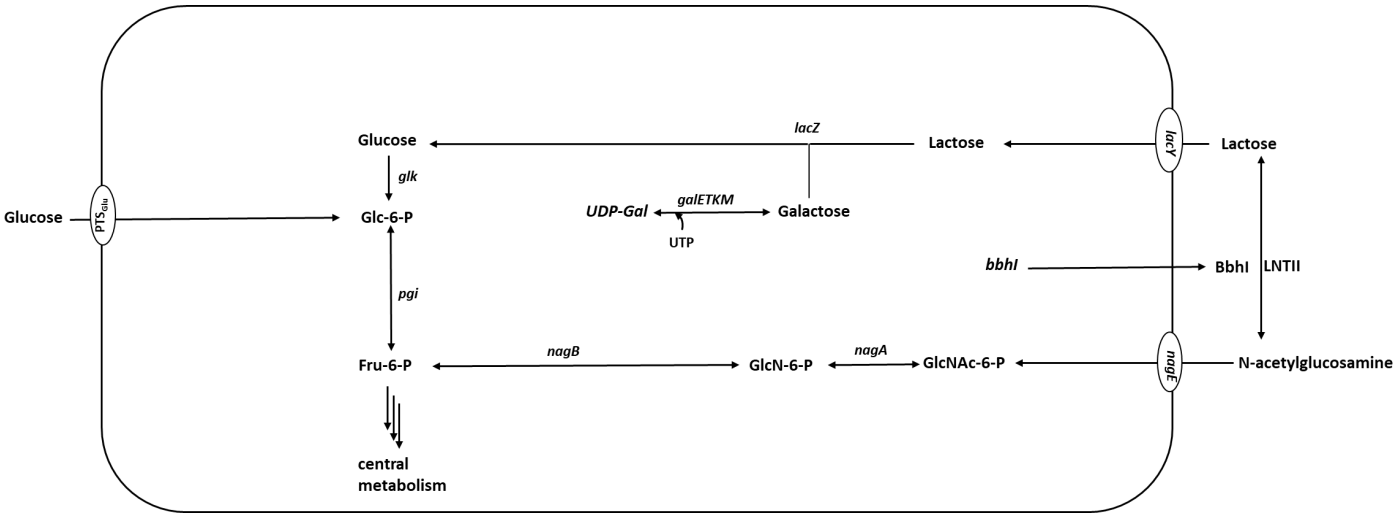


Figure 2. Degradation of Intermediates and By-products with DS-LNnT-JBT

Glc-6-P: glucose-6-phosphate, UDP-Gal: UDP-galactose, Fru-6-P: fructose-6-phosphate, LNT II: Lacto-*N*-triaose II, LNnT: Lacto-*N*-*neo*tetraose, GlcN-6-P: glucosamine-6-phosphate, GlcNAc-6-P: *N*-acetylglucosamine-6-phosphate.

2. Manufacturing

a. Quality

Lacto-*N*-*neo*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 an FDA-registered Food Facility (Registration # 1303109037512). Production also occurs at other Jennewein-qualified manufacturers that are GMP-, ISO-, and/or International Featured Standards Food 6.1-compliant via third party audits. Additionally, all raw materials, processing aids, and food contact substances are the same as those used to produce the 2'-fucosyllactose that is the subject of GRN 571, which received a "no questions" letter from FDA. None of the processing aids are recycled or reused.

b. Production

The production of LNnT begins with the propagation of precultures of PS-L*N*nT-JBT and DS-L*N*nT-JBT from the frozen glycerol stocks in medium containing glucose, fructose, sucrose, or glycerol or a combination thereof, as well as monoammonium phosphate, dipotassium phosphate trihydrate, potassium hydroxide, citric acid, magnesium sulfate heptahydrate, calcium chloride hexahydrate, as well as trace amounts of ammonium ferric citrate, manganese chloride tetrahydrate, cobaltus chloride hexahydrate, copper chloride dihydrate, boric acid, zinc sulfate

heptahydrate, sodium molybdate dihydrate, sodium selenite, and nickel sulfate hexahydrate. Each culture is then expanded in a pre-fermenter using the same fermentation medium and culture conditions. No antibiotics, inducers or inhibitors are added to the medium at any stage of the production process.

To generate LNnT, the expanded pre-fermenter culture of PS-L*N*nT-JBT is added to a main fermenter containing the same medium as the pre-culture and pre-fermenter cultures (Figure 3). The substrate lactose is then synthesized by the strain during fermentation or, alternatively, added to the growth medium prior to inoculation and converted to LNnT. While controlling pH and aeration, and monitoring the amount of lactose and LNnT in the fermentation medium by HPLC, fermentation proceeds until a majority of the lactose is converted to LNnT. DS-L*N*nT-JBT is then added to the main fermenter to degrade the residual lactose and intermediate LNT II. Commercial food-grade lactase may also be added to the main fermenter to enhance lactose degradation. When the amount of lactose, LNT II, and remaining monosaccharides (glucose, galactose, and *N*-acetylglucosamine) in the medium are exhausted (i.e., monosaccharides are undetectable and lactose and LNT II are below 3% (percent area) by HPLC), the biomass is separated from the fermentation medium by centrifugation, microfiltration and ultrafiltration, inactivated by heat or chemical treatment, and discarded. The oligosaccharide-containing supernatant is then processed in series of chromatographic, filtration, and decolorization steps to remove contaminating substances such as ions, trace elements, peptides, amino acids, DNA, and carbohydrate by-products. Decolorization is performed by using activated charcoal. The solution is concentrated either by nanofiltration or under a vacuum. Optionally further carbohydrate by-products can be removed by size-exclusion filtration steps or by chromatographic separation. The resulting concentrate is then filtered (i.e., 5 kDa and sterile filters) and dried (i.e., spray dried or similar methods) generating the LNnT powder. The powder is directly packaged into polyethylene-lined paper bags, sealed and stored under ambient conditions. Samples from the dried product are analyzed according to the requirements of the specifications (i.e., microbiological contamination, endotoxin content, heavy metal content, water content and LNnT purity). Batch analysis for carbohydrate content is conducted before the concentrate is filtered and dried. Additionally, the quality of the finished ingredient is controlled by monitoring all cultures for microbial contamination every 24 hr and one critical control point (CCP) located at the sterile filtration step, where pressure is monitored to ensure proper functioning of the filtration system.

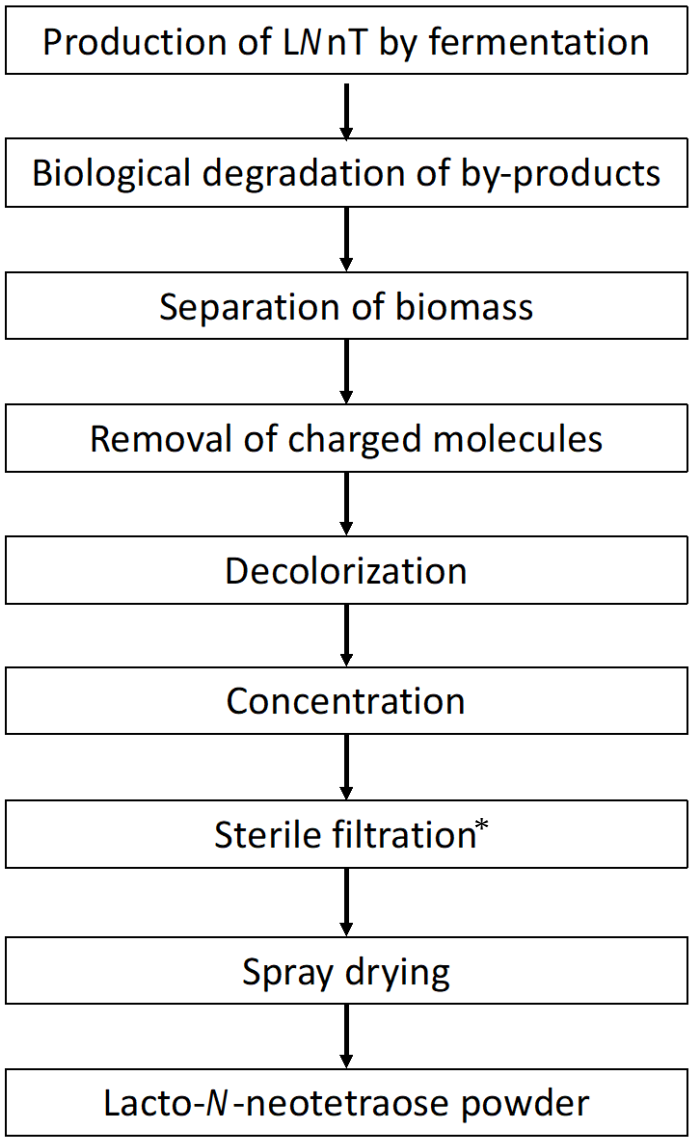


Figure 3. Flow Diagram of LNnT Production

All cultures are monitored for microbial contamination every 24 hr. The critical control point in the production process (denoted with an asterisk "*") is at the sterile filtration step where pressure is monitored continuously to ensure finished product sterility.

G. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. Product Specifications

To ensure a consistent food-grade product, each batch of LNnT powder is evaluated against a set of product specifications, which control the amount of LNnT, LNnT-related by-products, heavy metals, and microbes in the finished product (Table 3). Jennewein also has specifications in place to control the amount of DNA and endotoxin, which could result from the use of *E. coli* to manufacture the finished ingredient. Each parameter is measured using either compendial or internally validated methods at external DIN EN ISO/IEC 17025-accredited laboratories or Jennewein, respectively. Additionally, the specifications are similar to those in place for the subject of GRN 659, which was produced by fermentation using a metabolically engineered strain of *E. coli*. Data from five non-consecutive batches of LNnT powder show that the product is devoid of DNA and endotoxin and the manufacturing process produces a finished ingredient that reproducibly complies with the product specifications.

Table 3. Lacto-N-neotetraose Product Specifications and Batch Data

Parameter	Specification	Method	Batch Number				
			10916019	10916029	10916039	10916049	10916059
Chemical/Physical Parameters							
Appearance (Color)[4]	White to off-white	Visual	Complies	Complies	Complies	Complies	Complies
Appearance (Form)[4]	Powder to agglomerate	Visual	Complies	Complies	Complies	Complies	Complies
Structure[4]	Complies	NMR	Complies	Complies	Complies	Complies	Complies
Structure[4]	Complies	LC-MS	Complies	Complies	Complies	Complies	Complies
GMO detection[2]	Negative	qPCR	negative	negative	negative	negative	negative
LNnT[4]	≥ 92 % DW (w/w)	HPAEC-PAD	93.5	95.9	96.1	93.6	95.8
Lactose[4]	≤ 3.0 % (Area)	HPAEC-PAD	0.2	< LOQ	0.2	1.3	0.8
Lacto-N-triose II[4]	≤ 3.0 % (Area)	HPAEC-PAD	1.2	1.7	1.6	1.3	1.0
para-lacto-*N*-*neo*hexaose[4]	≤ 2.0 % (Area)	HPAEC-PAD	1.5	< LOQ	< LOQ	1.0	< LOQ
Sum of other human-identical saccharides[4]	≤ 8.0 % (Area)	HPAEC-PAD	3.5	2.4	2.1	2.8	2.4
Protein content[4]	≤ 100 µg/g	Nanoquant	1.8	0.5	0.7	1.1	1.0
Ash, sulfated[1]	≤ 0.4 % (w/w)	ASU L 39.00-9; 1981-04	< 0.10	0.16	< 0.05	0.24	0.12
pH[4]	5.0-7.0	20°C, 5% solution	6.9	6.8	6.4	6.9	6.9
Moisture[4]	≤ 9.0 % (w/w)	Karl Fischer Titration	6.3	7.8	8.6	8.6	7.1
Heavy Metals[1]							
Lead	≤ 0.02 mg/kg	ASU L 00.00-135 (ICP-MS): 2001-01	ND	ND	ND	0.014	0.013
Arsenic	≤ 0. 2 mg/kg	ASU L 00.00-135 (ICP-MS): 2001-01	ND	ND	ND	ND	ND
Cadmium	≤ 0.1 mg/kg	ASU L 00.00-135 (ICP-MS): 2001-01	ND	ND	ND	ND	ND
Mercury	≤ 0.5 mg/kg	ASU L 00.00-135 (ICP-MS): 2001-01	ND	ND	ND	ND	ND
Microbiological Parameters							
Standard Plate Count[1]	≤ 500 cfu/g	ISO 4833	<10	<10	< 10	< 10	< 10

Table 3. Lacto-N-neotetraose Product Specifications and Batch Data

Parameter	Specification	Method	Batch Number				
			10916019	10916029	10916039	10916049	10916059
Yeast and Mold[1]	≤ 100 cfu/g	ISO 21527-2: 2008-07	< 20	< 20	< 20	< 20	< 20
Bacillus cereus[1]	≤ 50 cfu/g	ASU L 00.00-33: 2006-09 Ber. 2006-12	<10	<10	< 10	< 10	< 10
Enterobacteriaceae[1]	≤ 10 cfu/g	ISO 21528-1: 2017-09	ND	ND	ND	ND	ND
Listeria monocytogenes[1]	Absent/25 g	ISO 11290-1: 1996-12	ND	ND	ND	ND	ND
Salmonella spp[1]	Absent/25 g	DIN EN ISO 6579-1:2017-07	ND	ND	ND	ND	ND
Cronobacter sakazakii[1]	Absent/10 g	ISO 22964: 2017-04	ND	ND	ND	ND	ND
Residual endotoxins[3]	≤ 10 EU/mg	Ph. Eur. 2.6.14	0.940	< 0.005	< 0.005	< 0.005	< 0.005

Abbreviations: NMR – nuclear magnetic resonance; LC-MS – liquid chromatography coupled with mass spectrometry; 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; ND = not detected; LOQ = limit of quantitation; DW – dry weight.

[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 quantitation (LOQ) = 0.05 mg/kg; Cadmium LOQ = 0.01 mg/kg; Mercury LOQ = 0.005 mg/kg; Lead LOQ = 0.01 ppm.

[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. Carbohydrate by-products with a percent area greater than 0.5% (limit of quantitation) are considered.

2. Other Quality Attributes

a. Elemental Analyses

During manufacturing, the production strains are cultured in medium containing a variety of elements to produce LNnT, and the LNnT is subsequently removed from the culture medium via a series of chromatographic, filtration, and decolorization steps to remove contaminating substances such as ions and trace elements. To confirm that the manufacturing process effectively removes the trace elements of the culture medium, Jennewein conducted elemental analyses on three batches of the finished ingredient. Iron, copper, selenium, and zinc were quantifiable whereas manganese, nickel, molybdenum and cobalt were all below the limit of quantitation (Table 4). These analyses will be conducted on an annual basis to ensure that the manufacturing process continues to produce a high-quality finish ingredient.

Table 4. Elemental Analysis of the LNnT[1]

Element	Method	Batch Number		
		10916059	10916019	10916039
Manganese (mg/kg)	ASU L 00.00-135 (ICP-MS)	<1.7	<1.7	<1.7
Iron (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.82	0.82	< 0.6
Copper (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.40	0.40	1.6
Molybdenum (mg/kg)	ASU L 00.00-135 (ICP-MS)	<0.06	<0.06	< 0.06
Nickel (mg/kg)	ASU L 00.00-135 (ICP-MS)	<0.02	<0.02	0.022
Zinc (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.24	0.24	< 0.13
Selenium (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.034	0.034	0.038
Cobalt (mg/kg)	PV-347 ICP-MS	<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; molybdenum LOQ = 0.06 mg/kg; nickel LOQ = 0.02 mg/kg; cobalt LOQ = 0.04 mg/kg; zinc LOQ = 0.13 mg/kg; iron LOQ = 0.6 mg/kg.

H. STABILITY OF LNnT POWDER

1. Stability of LNnT Powder

Because the subject of this GRAS determination is compositionally equivalent to the LNnT products that are subjects of GRN 547 and 659, it is reasonable to expect that the stability of the LNnT manufactured at Jennewein will be similar to the LNnT that are the subjects of GRN 547 and 659. Therefore, the bulk stability data on the chemically synthesized LNnT product that is the subject of GRN 547 are incorporated by reference. Additional stability data on the subject of GRN 547 was also included in GRN 659 and therefore incorporated by reference.

As specified in GRN 547, LNnT was packed in inner polyethylene bags packed in external polyethylene/aluminum/polyester triple layer foil bags and stored under accelerated (40°C/75% relative humidity) and ambient (25°C/60% relative humidity) conditions for 6 and 36 months, respectively. There were no changes in the organoleptic properties, no appreciable degradation of LNnT, no changes in the impurity profile, and no changes in the microbial parameters up to 6 months when stored under accelerated conditions, and for up to 36 months under ambient conditions. In GRN 659, the Notifier also stated that there were no changes in organoleptic properties of LNnT, no appreciable degradation of LNnT, no changes in impurity profile, and no alterations in the microbiological quality of the ingredient following storage for up to 2 years under accelerated conditions and for up to 5 years under ambient storage conditions.

These data therefore support a shelf-life of up to 2 years under accelerated (40°C/75% relative humidity) and 5 years under ambient (25°C/60% relative humidity) conditions for the Jennewein-manufactured LNnT. Additionally, the identity of the finished product manufactured by Jennewein has been confirmed to be LNnT and the moisture content is below 9%, hence the likelihood of microbial growth in the product is low.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

The intended effect of adding LNnT to non-exempt term infant formula, conventional foods, and beverages is to increase LNnT intake in formula-fed infants and the general population and promote the growth of beneficial bacteria, including, but not limited to bifidobacteria.

B. HISTORY OF EXPOSURE

Humans are exposed to LNnT either through the ingestion of breast milk and/or products containing synthetic forms LNnT.

As summarized in GRN 547 and 659, LNnT is one of the most abundant human milk oligosaccharides. Although the levels of LNnT in human milk varies with ethnicity, Secretor and Lewis-blood group status, lactation period, term vs preterm birth (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 average LNnT concentration in pooled mature breast milk (from full-term birth mothers at approximately lactation days 5 to 100) ranges from 110 and 630 mg/L (reviewed in Section IV.B.1 of GRN 547). Studies published since the filing of GRN 547 have also reported similar results (Austin et al., 2016; Sprenger et al., 2017; Williams et al., 2017; Austin et al., 2019). Because a 6.5 kg infant consumes approximately 1 L of breast milk/day (Davies et al., 1994; Hester et al., 2012), LNnT intake from mature breast milk ranges from 20 to 100 mg/kg body weight/day. Therefore, the background exposure to LNnT from human milk serves as the safe range for Jennewein's LNnT.

In the United States, two synthetic forms of LNnT products are GRAS for use in term non-exempt infant formula and conventional foods. The first GRN, GRN 547, was filed in 2014 for a chemically synthesized LNnT with intended use levels of 0.6 g/L in infant formula and up to 20 g/kg in selected conventional foods. The second GRN, GRN 659, was filed in 2016 for an LNnT product manufactured by fermentation with the same use levels as specified in GRN 547. LNnT is also a Novel Food in the European Union and has been available since 2018.

C. INTENDED USES

Jennewein intends to use LNnT powder as a substitute for other forms of LNnT. The intended uses are therefore the same as those specified in GRN 659, which are term non-exempt infant formula, and selected conventional foods, and beverages (Table 5). Therefore, the

intended uses and corresponding use levels from GRN 659 are incorporated by reference (see Section I.D of GRN 659). The intended use levels will not exceed 0.6 g/L reconstituted infant and toddler formula, and 20 g/kg in selected conventional foods and beverages.

Table 5. Intended Uses of LNnT[1]

Food Category	Proposed Food Uses	Maximum Proposed Use Level (g/kg or g/L)
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	2.5
	Sports, Isotonic, and Energy Drinks	0.58
Dairy Product Analog	Imitation Milks	0.58
	Non-Dairy Yogurt	2.67
Infant and Toddler Foods	Non-exempt Term Infant Formulas	0.6
	Toddler Formulas	0.6
	Other Baby Foods for Infants and Young Children	3.0
	Other Drinks for Young Children	0.58
Grain Products and Pastas	Meal Replacement Bars for Weight Reduction	20.0
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized Milk[a]	0.58
Milk Products	Buttermilk	0.58
	Flavored Milk	0.58
	Milk-Based Meal Replacement Drinks, for Weight Reduction	2.5
	Yogurt	2.67
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	0.58

[1]Obtained from Section 1.D, GRN 659.
[a]Milk is a standardized food in the United States. When milk is fortified with LNnT, it will then be classified as a milk product.

D. ESTIMATED DAILY INTAKE

Because Jennewein intends to use LNnT powder as a substitute for other forms of LNnT currently marketed in the United States and the intended uses and use levels are the same as those specified in GRN 547 and 659, which received "no questions" letters from FDA, the resulting estimated daily intake of LNnT from infant formula and selected conventional foods and beverages will not exceed those associated with other forms of LNnT. Therefore, the LNnT infant formula exposure assessment provided in GRN 547 and the LNnT selected conventional food and beverage exposure assessment provided in GRN 659 are incorporated by reference and briefly summarized below.

1. Infant Formula

Using the 2011-2012 National Health and Nutrition Examination Surveys (NHANES), the mean and 90th percentile intake of LNnT from infant formula were determined to be 510 and 730 mg/day (83.2 and 133.9 mg/kg bw/day), respectively, for infants aged 0 to 6 months (GRN 547, Sections IV.A.1 and IV.A.2, pages 17-20). For infants 7 to 12 months of age, the estimated mean and 90th percentile all-user intakes of LNnT from infant formulas were determined to be 420 and 660 mg/day (48.5 and 79.5 mg/kg bw/day), respectively (Sections IV.A.1 and IV.A.2 of GRN 547 ang GRN 659).

2. Selected Conventional Foods and Beverages

Using the 2011-2012 NHANES, the exposure assessment conducted in GRN 659 identified approximately 85.2 % of the US population as potential consumers of LNnT-containing foods (GRN 659, Section IV.A). All-user mean and 90th percentile LNnT intakes from the intended uses and use levels was estimated to be 304 and 646 mg/day (8.1 and 16.8 mg/kg bw/day), respectively. The highest consumers were toddlers with mean and 90th percentile intakes of 514 and 901 mg/day (38.4 and 67.7 mg/kg bw/day), respectively. The lowest users at the mean were elderly adults who would consumed 243 mg/day (3.4 mg/kg bw/day). The lowest uses at the 90th percentile were female adults at child-bearing age who would consume 526 mg/day (8.0 mg/kg bw/day).

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 LNnT according to the specified conditions of use in non-exempt term infant formula and conventional foods and beverages is based on published studies that quantitate the levels of LNnT in human milk (see Section III.B), published and unpublished toxicological studies in neonatal and juvenile rats summarized in GRN 547 and 659, chemical equivalence to the LNnT products that are the subjects of GRN 547 and 659, and the data demonstrating that the LNnT produced by Jennewein is structurally identical to LNnT from human milk.

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 (reviewed in Bode et al., 2012). 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).

Non-fucosylated neutral HMOs, including LNnT, constitute 42-55% of the total HMO fraction in breast milk (Van Niekerk et al., 2014; Smilowitz et al., 2014). LNnT is among the most abundant HMOs, as reported in ≥ 15 studies from ≥ 6 independent research groups. Although its absolute concentration varies with ethnicity, Secretor and Lewis-blood group status, and lactation period, LNnT is generally highest in colostrum (up to 550 mg/L at days 3-10 post-partum) and lowest in mature breast milk (200 mg/L at days 31-452 post-partum) (Erney et al., 2000). Based on available data, the range of average concentrations of LNnT in pooled, mature breast milk (from full-term birth mothers at approximately lactation days 5-100) has been reported to be 110-630 mg/L (See Section III.B).

Because human milk is the reference standard for infant nutrition, infant formula manufacturers look to mimic the composition of human milk in their formulas as much as possible. Manufacturing HMOs and including them in infant formula on a commercial scale, however, has not been feasible until recently. Thus, infant formula manufacturers have resorted to supplementing their formulas with other synthetic and/or plant-based non-digestible oligosaccharides to confer the prebiotic effects of HMOs. These other oligosaccharides include galactooligosaccharides (GOS), polydextose, oligofructose, long-chain inulin, and fructooligosaccharides (FOS) (GRN 233, 285, 286, 334, 392, 477, 484, 489, 495, 518, 537, 569, 576, 620, 721, 729, 797). Galactooligosaccharides (GOS), in specific, are GRAS for use in infant formula at 7.2 g/L and their use is supported by extensive preclinical and clinical data (GRN 233, 285, 286, 334, 484, 489, 495, 518, 569, 620, 721, 729). Although GOS promotes the growth of bacteria typically found in the breast-fed infant gastrointestinal tract, they are not natural components of human milk. Therefore, supplementing infant formula with synthetic oligosaccharides that are identical to those present in human milk provides a formula that more closely resembles both the composition and activities of human milk. Although this calls into question the addition of selected HMOs opposed to a mixture of the almost 200 HMOs in infant formula (Milani et al., 2017), both the types and amounts of HMOs vary in breast milk from one mother to another (see Section III.B), there is no clear and/or consistent association between the levels of structurally different HMOs in breast milk and adverse outcomes on infant growth and health (Aldetete et al., 2015 Azad et al., 2018; Gridneva et al., 2019; Kuntz et al., 2019; Larsson et al., 2019; Sprenger et al., 2017; Vandenplas et al., 2018), and non-digestible carbohydrates that are not components of human milk are GRAS for use in infant formula and conventional foods at levels greater than those intended for LNnT (GRN 233, 285, 286, 334, 392, 477, 484, 495, 518, 537 569, 576, 623, 620, 797). Thus, it is reasonable to expect that supplementing infant formula and selected conventional foods with a synthetic form of LNnT would not pose risks to LNnT consumers.

In the United States, two LNnT preparations have been determined GRAS for use in non-exempt infant formulas for term infants, as well as a variety of food products (GRN 547; GRN 659). The subject of GRN 547 was the first LNnT-containing product to be determined GRAS. It is chemically synthesized and structurally identical to the LNnT found in breast milk. Additionally, its use as a food ingredient is supported by use levels that are within the range of LNnT levels found in breast milk, a variety of genotoxicity and subchronic toxicity studies in juvenile rats published by Coulet et al. (2013), and corroborative clinical studies conducted in infants and healthy adults. The second LNnT product to be determined GRAS is the subject of GRN 659, which is manufactured by fermentation using a genetically engineered strain of *E. coli* and structurally identical and chemically equivalent to the subject of GRN 547. Although the safety of the fermentation-produced LNnT is supported by unpublished genotoxicity and

subchronic toxicity studies, because the product is structurally identical and chemically equivalent to the subject of GRN 547, the genotoxicity and subchronic toxicity studies published by Coulet et al. (2013) were considered to be pivotal for determining the safe use of the fermentation-produced LNnT. Importantly, because the LNnT produced by Jennewein is structurally identical and chemically equivalent to the LNnT products that are the subjects of GRN 547 and 659 and will be used as a substitute for the other forms of LNnT that are GRAS, the published and unpublished studies reviewed in GRN 547 and 659, as well as the studies that have been published since the filing of GRN 659, support the safe use of the Jennewein product in infant formula, selected conventional foods, and beverages.

LNnT is not genotoxic and two Organization for Economic Cooperation and Development (OECD)-compliant 90-day toxicology studies in neonatal rats each determined the no observed adverse effect level (NOAEL) for LNnT to be at least the maximum tested dose of 5000 mg/kg bw/day (Coulet et al., 2013; GRN 659). Additionally, based on the results reported in the OECD-compliant 90-day study published by Coulet et al. (2013), the European Food Safety Authority considers the NOAEL for LNnT to be 2500 mg/kg bw/day (EFSA Panel on Dietetic Products, 2015). In contrast, the 90th percentile EDIs resulting from the ingestion of LNnT from infant formula are 730 and 660 mg/day (133.9 and 79.5 mg/kg/bw/day) for 0 to 6 and 7 to 12 month-old infants, respectively, and 646 mg/day (16 mg/kg bw/day) for the 90th percentile consumers (age 2 and up) of LNnT-containing selected conventional foods and beverages. Thus, the NOAELs that were established in the 90-day toxicology studies published by Coulet et al. (2013), cited in GRN 659, and established by EFSA, are supportive of the EDIs for LNnT.

Based on these data, there is reasonable certainty that the use of Jennewein's LNnT per the intended uses and use levels is of no harm to consumers. Jennewein's LNnT is therefore GRAS as an ingredient in conventional food and beverages, as well as non-exempt, term infant formula at the intended use levels.

A. CHEMICAL EQUIVALENCE OF LNnT PRODUCTS

Analysis of the carbohydrate by-product profiles of three batches Jennewein's LNnT using high performance liquid chromatography (HPLC) coupled with a Corona Charged Aerosol Detector shows that the LNnT that is subject of this GRAS Notice contains residual quantities of Gal-LNnT, Glc-LNnT, GlcNAc-GlcNAc-lactose tetraose, Glc-lactose, and Glc-Gal tetraoses (Table 6), as well as D-lactose, lacto-N-triose II (LNT II), and *para*-lacto-*N-neo*hexaose (para-LNnH) (Table 3). In contrast, the LNnT that is the subject of GRN 659 contains D-lactose, LNT II, para-LNnH, an LNnT fructose isomer, lactulose, Gal-LNnT, GlcNAc-LNnT, Gal-GlcNAc-Gal, 2-Gal-trehalose, and a p-LNnH fructose isomer. Importantly, the levels of lactose, LNT II

and para-LNnH in both LNT products are controlled by comparable product specifications (compare Table 3 in this Notice with Table II.C.2-1 of GRN 659). Additionally, the LNnT fructose isomer, lactulose, 2-Gal-trehalose, and p-LNnH fructose isomer present in the subject of GRN659 are not expected in the subject of this Notice due to Jennewein's manufacturing process. Because Jennewein's product specifications (LNnT, LNT II, para-LNnH, ash, and moisture) account for 100 % of the finished product on a dry weight basis, the Gal-LNnT, Glc-LNnT, GlcNAc-GlcNAc-lactose tetraose, Glc-lactose, and Glc-Gal tetraoses present in the finished product would occur only in minute quantities and therefore not affect the product's quality and/or safety.

Table 6. Carbohydrate By-Product Profile of Lacto-N-neotetraose[1]

Carbohydrate By-product	Batch Number		
	10916019	10916029	10916039
Gal-LNnT (% Area)	0.63	0.51	0.51
Glc-LNnT (% Area)	0.82	0.53	0.53
Tetraose (GlcNAc-GlcNAc-Lac) (% Area)	1.68	1.02	0.99
Triaose (Glc-Lac) (% Area)	1.75	1.66	0.50
Tetraose (Glc/Gal-Lac)[2] (% Area)	0.59	0.52	0.50
Unspecific impurties	1.01	0.59	0.69

Gal, galactose; Glc, glucose; GlcNAc, N-acetylglucosamine; Lac, lactose; LNnT, lacto-*N*-*neo*tetraose
[1]Determined by Jennewein Biotechnologie using HPLC and Corona Charged Aerosol Detector. Carbohydrate by-products with a percent area greater than 0.5% (limit of quantitation) are considered.
[2]Linear combinations of two glucose and/or galactose moieties bound to a terminal lactose.

B. SAFETY OF THE PRODUCTION ORGANISMS

The subject of this GRAS Notification is produced using two genetically engineered organisms, PS-L*N*nT-JBT and DS-L*N*nT-JBT, which are derived from the host organism *E. coli* BL21(DE3). *E. coli* BL21(DE3) is routinely used in the industry for the manufacture of food and pharmaceutical ingredients, and thoroughly described in GRN 485 (pg. 15-18) and GRN 571 (Appendix K) in which genetically engineered strains of *E. coli* BL21(DE3) are used to produce BbgIV *beta*-galactosidase and 2'-fucosyllactose (GRN 485; GRN 571). Importantly, both GRNs received "no questions" letters from the FDA.

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 organisms are chromosomally integrated, result in the expression of well-characterized proteins, and the organisms are removed from the finished ingredient during manufacturing, there is reasonable certainty that neither the host organism nor the genetic modifications to the production strains pose safety risks to the consumer.

C. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

The ADME of LNnT is thoroughly reviewed in GRN 547, pg. 25-27, and further summarized in GRN 659, pg. 31-32. These reviews are therefore incorporated by reference. In summary, HMOs, including LNnT, are highly resistant to the digestive enzymes of the gastrointestinal (GI) tract and only small amounts are absorbed intact (EFSA Panel on Dietetic Products, 2015). In vitro studies have shown that <5% of ingested HMOs is digested in the GI tract and in vivo studies in infants and rats have reported that 1-2% of the total amount of ingested HMO is excreted unchanged in urine (Goehring et al., 2014; Ruhaak et al., 2014; Santos-Fandila et al., 2014; Dotz et al., 2014; Obermeier et al., 1999; Rudloff and Kunz, 2012; Rudloff et al., 2006; Rudloff et al., 2012; Rudloff et al., 1996; Chaturvedi et al., 2001; Goehring

et al., 2014; Ruhaak et al., 2014; Gnoth et al., 2000; Engfer et al., 2000). Similar results have been reported for LNnT (Chaturvedi et al., 2001; Goehring et al., 2014). Although the exact mechanisms by which HMOs are absorbed are still unclear, in vitro studies suggest that neutral HMOs, such as LNnT, may be absorbed by receptor-mediated transcytosis and paracellular transport. The remaining unabsorbed HMOs, including LNnT, are then either fermented by the intestinal microbiota or excreted unchanged in the feces.

Since the filing of GRN 659, Vazquez et al. (2017) published a pharmacokinetic study that evaluated the kinetics and metabolic fate of absorbed LNnT in rats. Either 0.29 or 1.45 g LNnT/kg were administered by gavage, and the levels of LNnT, lactose, and fucose, sialic acid, 2'-fucosyllactose, 6'-sialylactose, and 3'-sialyllactose levels were quantitated in serum and urine over the course of 300 minutes by ultra-performance liquid chromatography coupled mass spectrometry (LC-MS). LNnT was not detected in the serum at baseline. Following gavage, serum LNnT levels increased to a maximum of approximately 2.5 μg/ml by 60 minutes after the administration of 0.29 g LNnT/kg and approximately 4.5 μg/ml at 180 minutes after the administration of 1.45 g LNnT/kg. The serum levels then declined, approaching baseline by 300 minutes. Urinalysis revealed that the LNnT levels were low at baseline and increased between 90 and 120 min post gavage. Additionally, except for lactose, which increased 30 minutes after the LNnT gavage, the serum levels of the other carbohydrates detected by the LC-MS method were not affected by the administration of LNnT. These data confirm the results of previous studies that small amounts of LNnT are absorbed and metabolized at least in part to lactose either prior to and/or during absorption.

Importantly, because the LNnT that is the subject of this GRAS Notification is structurally identical to the LNnT found in breast milk and the resulting estimated daily intake approximates the typical LNnT intake from breast milk (see Section III.B), there is reasonable certainty that the absorption, distribution, metabolism, and excretion of LNnT ingested from the intended uses will mimic that from breast milk.

D. TOXICOLOGY STUDIES

The studies that have evaluated the toxicity of LNnT products include a battery of published and unpublished genotoxicity and sub-chronic toxicity studies, which are extensively summarized in GRN 547 and 659. Because Jennewein's LNnT is structurally identical to the LNnT that is the subject of GRN 547 and 659 and Jennewein's LNnT-containing product is chemically equivalent to the subjects of both GRN 547 and 659, the published and unpublished toxicity studies that support the safe use of the subjects GRNs 547 and 659 also support the safe use of Jennewein's LNnT in infant formula, and conventional foods and beverages. Therefore, the published and unpublished genotoxicity and subchronic toxicity studies extensively summarized in GRN 547 and 659 are incorporated by reference and briefly summarized below.

Additionally, no new studies were identified during the literature search conducted on December 19, 2019 that would suggest that LNnT may be unsafe for consumption.

1. Genotoxicity Studies

The genotoxicity studies that support the safe use of LNnT in infant formula and conventional foods include a bacterial reverse mutation test (Ames test), in addition to two Organization for Economic Cooperation and Development (OECD)-compliant bacterial reverse mutation tests, an OECD-compliant in vitro mammalian cell gene mutation test in L5178Y tk+/- mouse lymphoma cells, an OECD-compliant in vitro micronucleus test conducted in peripheral human lymphocytes, and an *in vitro* mutagenesis study. All OECD-compliant studies were conducted with and without metabolic activation. The OECD-compliant bacterial reverse mutation and *in vitro* mammalian cell gene mutation test in L5178Y tk+/- mouse lymphoma tests were conducted with the chemically synthesized LNnT that is the subject of GRN 547 and published by Coulet et al. (2013). The other OECD-compliant bacterial reverse mutation test and *in vitro* micronucleus test in peripheral human lymphocytes were conducted with LNnT that is manufactured by fermentation using a genetically engineered strain of *E. coli*, the subject of GRN 659, and have not been published. The remaining non-OECD-compliant bacterial reverse mutation test and the *in vitro* mutagenesis study in animal cells were conducted with an LNnT-containing product manufactured by yeast fermentation and published by Prieto et al. (2005). Because all of these studies are summarized in detail in Section IV.B.5 of GRN 547 (pg. 34-35) and Section IV.E.2 of GRN 659 (pg. 36-37), they are all incorporated by reference and summarized below.

Coulet et al. (2013) reported that chemically synthesized LNnT, with a purity of 98.9%, was not mutagenic up to the limit concentration of 5000 µg/plate in a bacterial reverse mutation assay using five strains of *S. typhimurium*, in the presence and absence of metabolic activation (S9). The chemically synthesized LNnT also did not produce statistically or biologically significant increases in the frequency of mutations up to 4250 µg/mL in an *in vitro* mammalian cell gene mutation assay using L5178Y $TK^{+/-}$ mouse lymphoma cells.

In the unpublished studies summarized in GRN 659, LNnT manufactured via fermentation, with a purity of 94 %, was not mutagenic up to the limit concentration of 5000 µg/plate in the bacterial reverse mutation assay using five strains of *S. typhimurium* and *E. coli* WP2uvrA both in the presence and absence of metabolic activation (S9) and did not produce statistically or biologically significant increases in the frequency of mono- or bi-nucleated cells with micronuclei in the short-term (3 h, ±S9) and long-term (24 h, −S9 only) *in vitro* micronucleus assays using cultured peripheral human lymphocytes.

Prieto et al. (2005) also reported that an LNnT-containing product of unknown purity manufactured by yeast fermentation was not mutagenic in a bacterial reverse mutation test or an *in vitro* mutagenesis study in animal cells, although no experimental details or data were provided. Taken together, these results demonstrate that LNnT is not genotoxic.

2. Subchronic Toxicology Studies

The toxicity of LNnT has been evaluated in numerous studies including a 14-day dose-range finding study in neonatal rats, a 28-day repeated dose study in neonatal rats, an OECD-compliant 28-day repeated-dose study in neonatal rats, two OECD-compliant 90-day subchronic oral toxicity studies conducted in neonatal rats, and a 4-month dietary study in juvenile rats GRN 659; Coulet et al., 2013; Prieto, 2005). The 14-day dose-range finding study, the OECD-compliant 28-day repeated-dose study, and one of the OECD-compliant 90-day subchronic oral toxicity studies were published by Coulet et al. (2013) and conducted with a chemically synthesized form of LNnT with a purity of 98.9% that is the subject of GRN 547. The second OECD-compliant 90-day subchronic oral toxicity study was conducted with an LNnT manufactured by fermentation using a genetically engineered strain of *E. coli* with a purity of 94 %, was not published and is summarized in GRN 659. The 28-day repeated dose and 4-month dietary studies in neonatal/juvenile rats were published by Prieto et al. (2005) and used an LNnT product of unknown purity manufactured by yeast fermentation. In both OECD-compliant 90-day toxicology studies, the NOAELs were determined to be a least 5000 mg/kg bw/day, the highest doses tested. Importantly, because the subject of this GRAS Notice is structurally identical and compositionally similar to the subject of GRN 547, the OECD-compliant 90-day subchronic oral toxicity conducted on the LNnT that is the subject of GRN 547 and published by Coulet et al. (2013) is the pivotal study that supports the safe use of Jennewein's LNnT in infant formula and selected conventional foods and beverages. All of the remaining studies corroborate the results reported by Coulet et al. (2013) and support the safe use of the LNnT that is the subject of this GRAS notification. Because all studies have been extensively reviewed in GRN 549 and 659, they are all incorporated by reference and briefly summarized below.

In a 14-day dose-range finding, an OECD-compliant 28-day repeated-dose, and an OECD-compliant 90-day subchronic oral toxicity studies published by Coulet et al. (2013), either a vehicle control or a chemically synthesized form of LNnT with a purity of 98.9% was administered by oral gavage at doses of 1000, 2500, and 5000 mg/kg body weight/day to neonatal rats (post-natal day 7) (see Section IVB.5.1 of GRN 547, pg. 28-33). All studies also included an additional group receiving a daily gavage delivering 5000 mg/kg body weight/day oligofructose (OF). In the 14-day study, no mortality and no abnormalities in clinical signs, body weights, and necropsy were observed following LNnT administration at any dose. Thus, the highest dose of 5000 mg/kg body weight/day was considered suitable for the longer-term studies. In the subsequent 28-day oral toxicity study, which included a 14-day recovery period for a

subset of animals, LNnT at the highest dose of 5000 mg/kg body weight/day resulted in soft, liquid, yellow-colored feces and erythema on the urogenital area in a few animals up until the period of weaning (day 13/14); these effects occurred more frequently in animals receiving OF. No other toxicologically relevant clinical observations, ophthalmological abnormalities, differences in body weight and body weight gain, feed and water intake, hematology, clinical chemistries, urinalysis, organ weights, macroscopic or microscopic findings, mortality or morbidity were attributed to LNnT administration. Finally, in the subchronic 90-day oral toxicity study, which included a 28-day recovery period for a subset of animals, a few animals receiving 5000 mg/kg body weight/day LNnT again showed yellow liquid feces and reddening of the urogenital region; this was significantly more frequent in animals receiving 5000 mg/kg OF. No clinically or toxicologically significant differences in body weights, body weight gain, hematology, clinical chemistry, organ weights, macroscopic or microscopic findings, mortality or morbidity were attributed to LNnT administration. Based on these results, the NOAEL for the chemically synthesized LNnT was 5000 mg/kg body weight/day, the highest dose tested.

In the corroborative unpublished OECD-compliant 90-day subchronic oral toxicity study summarized in GRN 659, either LNnT produced via fermentation with a purity of 94 % at 0, 1000, 2500, or 5000 mg/kg body weight LNnT or 5000 mg/kg body weight/day fructooligosaccharide (FOS) were administered by oral gavage (see Section IV.E.1 of GRN 659, pg. 34-35). A subset of animals was maintained for a 28-day recovery period following test article administration. No test-related mortalities were observed, and no toxicologically relevant effects on ophthalmology, body weight, body weight gain, feed consumption, clinical signs, hematology, clinical chemistry, urinalysis, organ weights, macroscopic or microscopic findings were noted when comparing the LNnT and control groups. Based on these results, the NOAEL for fermentation produced-LNnT in these studies was determined to be 5000 mg/kg body weight/day, the highest dose tested.

Prieto et al. (2005) conducted a 28-day and a 4-month study in rats using an LNnT-containing product manufactured using a yeast fermentation process (see Section IVB.5.2 of GRN 547, pg. 23-34). In the 28-day study, 12 litters (5/sex/litter) of 15 days old pups were administered control or 10, 200, or 400 mg LNnT/kg body weight/day (purity not reported) via gavage. Parameters evaluated included urinalysis, hematology, fecal analysis, and gross pathology. Although Prieto et al. reported that there were no significant differences in any of the parameters measured among groups, no data were provided to confirm their conclusions. In the 4-month study, 31- to 37-day-old rats (sex, strain, and number not reported) were fed diets containing 1 or 5% of LNnT for 4 months. Whether a control group was included in this study was not reported and although Prieto et al. stated that detailed clinical chemistry and histopathological examinations were conducted, no further details or data were provided. Prieto

et al. concluded that no test article-related adverse effects or macroscopic and microscopic changes were observed following LNnT administration.

E. CLINICAL STUDIES

The clinical studies that support the safe use of LNnT in infant formula, conventional foods and beverages include one study published by Prieto et al. (2005) that was extensively summarized in GRN 547 (pg. 35-36), two studies published by Puccio et al. (2017) and Elison et al. (2016), which were extensively summarized in GRN 659 (pg. 38-41), and one study that has been published since GRN 659 was filed with FDA, Nowak-Wegrzyn et al. (2019). Because Jennewein's LNnT is structurally identical to the LNnT that is the subject of GRN 547 and Jennewein's LNnT-containing product is chemically equivalent to the subjects of both GRN 547 and 659, the studies published by Prieto et al. (2005), Puccio et al. (2017), and Elison et al. (2016) support the safe use of Jennewein's LNnT product in infant formula, conventional foods, and beverages, and are incorporated by reference and briefly summarized below. Overall, the ingestion of 1 g/L of LNnT was reported to be well-tolerated in infants and 20g/d in adults.

Prieto et al. (2005) administered either a control formula or a formula containing 220 mg/L LNnT in a double-blind, randomized, placebo-controlled study involving 228 healthy infants (6-24 months old). The subjects were monitored for 16-weeks during which biweekly oropharyngeal swabs were conducted for monitoring *Streptococcus pneumoniae* colonization. Growth and ear health were also monitored. There was no significant difference between the two groups in *S. pneumoniae* colonization rate and LNnT was well-tolerated and had no significant effects on growth or ear health.

Puccio et al. (2017) administered a standard formula or a formula supplemented with 0.5 g/L LNnT (production method not specified) and 1.0 g/L 2'-fucosyllactose (2'-FL) to healthy full-term infants (0-6 months old) from the time of enrollment until 12 months of age in a randomized, blinded, controlled, multi-center, parallel study. Safety parameters including weight gain, body length, head circumference, digestive tolerance, formula compliance, and morbidity including adverse events were monitored. One hundred seventy-five infants enrolled in the study. Infants receiving the test formula did not differ from control with regard to weight, length, head circumference, body mass index, or digestive tolerance. Subjects receiving LNnT/2'-FL-supplemented formula experienced bronchitis less frequently through 4 months ($p=0.010$), 6 months ($p=0.005$) and 12 months ($p=0.004$), receive less antipyretics through 4 months ($p=0.032$) and antibiotics through 6 months ($p=0.047$) and 12 months ($p=0.016$), and experienced fewer lower respiratory tract infections through 12 months ($p=0.027$) compared to the standard formula. The study concluded that formula containing LNnT and 2'-FL was safe, well-tolerated, and supported age-appropriate growth.

Elison et al. (2016) administered single daily doses of LNnT, 2'-FL, or a combination of LNnT and 2'-FL at a ratio of 2:1 at 5, 10, or 20 g/day to 100 healthy adult volunteers for 2 weeks in a randomized, placebo-controlled, double-blind, parallel-design study. Glucose was used as a placebo. Safety and tolerance parameters were monitored including adverse events, standard hematological and biochemical parameters, fecal sampling, GI symptoms, and changes in bowel habits. All adverse events reported during the study were judged to be "mild" and there were no cases of premature discontinuation from the trial due to adverse events. No clinically relevant changes in hematological or biochemical parameters were observed. LNnT was well-tolerated, and no changes in bowel habits versus control were noted. Subjects receiving 10 or 20 g/day LNnT reported significantly higher incidence of passing gas compared to control subjects. Overall, these findings support that LNnT is safe and well-tolerated in healthy adults.

Nowak-Wegrzyn et al. (2019) conducted a cross-over double-blind, placebo-controlled, food challenges (DBPCFC) in sixty-seven infants 2 months to 4 years of age with documented cow milk protein allergy, who received either a control formula (hypoallergenic, whey-based, extensively hydrolyzed formula without HMOs) or formula supplemented with 0.5 g/L LNnT and 1.0 g/L 2'-FL. The test and control products were administered in a random order, with the second challenge occurring 2-7 days after the first. For subjects ≤ 1 year of age, the initial dose was a lip smear with the assigned infant formula, followed by oral doses of 5, 10, 20, 30, 30, 35, and 50 mL at 10–15 min intervals (total volume 180 mL). For subjects > 1 year of age, the initial dose was a lip smear, followed by 5, 10, 25, 45, 45, 45, and 65 mL orally at 10–15 min intervals (total volume 240 mL). Data were included in the analysis if subjects had consumed a minimum of 100 mL of formula, and a minimum observation period of 1 h after the final dose was required. Any allergic signs attributable to the challenge formula were documented. Subjects successfully passing both challenge sessions proceeded to a one-week open challenge with the test formula to assess tolerance and confirm the absence of any delayed allergic reactions. The parents or legal guardians were given the test formula and instructed to administer a minimum of 240 mL daily, for a period of one week (7–9 days). Daily formula intake as well as clinical parameters including daily stool frequency and quality, frequency of flatulence, frequency of spitting-up and/or vomiting, potential allergic signs, and other adverse or serious adverse events were reported. Sixty-one infants (age 24.1 ± 13.2 months) completed the challenge sessions in accordance with the protocol and proceeded to the open challenge period. The six that did not complete the study were either unable to consume enough formula (n=2), outside the desired age range, withdrew before completing the second DBPCFC, or erroneously completed both DBPCFC with the test formula. Fifty-five (90.2%) subjects consumed a minimum of 240 mL (average 302 ± 161 mL) of the test formula/day over the entire study period. Two patients reported gastrointestinal symptoms. One subject vomited on Day 1 of the home challenge, but completed the study without further problems. Another patient developed diarrhea on the last day of the challenge, which the site investigator attributed to gastroenteritis. The episode resolved

after 4 days. Otherwise, no significant gastrointestinal signs were reported. There were no reactions that warranted early discontinuation of the open formula challenge. Also, no serious adverse events occurred during the study. The results indicate that LNnT does not provoke allergic responses in CMA infants.

Taken together the results reported by Prieto et al. (2005), Puccio et al. (2017), Elison et al. (2016), and Nowak-Wegrzyn et al. (2019) indicate that the ingestion of LNnT-containing products is generally well tolerated and thus, there is reasonable certainty of no harm.

F. OTHER CORROBORATIVE INFORMATION

An additional corroborative study was retrieved from the published literature where a mixture of LNnT, 2'-FL, 6'-sialyllactose, 3'-sialyllactose, and free sialic acid or a mixture of galactooligosaccaharides and fructooligosaccharides were evaluated for their effects on systemic and gastrointestinal immune parameters in noninfected and rotavirus-infected pigs (Comstock et al., 2017). Specifically, colostrum-deprived newborn pigs were fed formula with or without 4 g/L of a mixture of HMOs (35% LNnT, 40% 2'-FL, 10% 6'-sialyllactose, 5% 3'-sialyllactose, and 10% free sialic acid), or 3.6 g/L short-chain galactooligosaccharides (GOS) and 0.4 g/L long-chain FOS. On day 10, half of the pigs were infected with the porcine rotavirus strain OSU. Regardless of infection status, the HMO-fed pigs had nearly twice as many natural killer cells, 36% more effector memory T cells, and 5 times as many basophils compared to control pigs. Pigs receiving prebiotics (GOS/FOS) had cell populations intermediate between formula- and HMO-fed pigs. In non-infected pigs, the HMO mix resulted in twice as many interferon gamma (IFNγ)-producing cells compared to formula-fed animals. Formula also resulted in more macrophages and mature dendritic cells, but fewer immature dendritic cells than did HMOs. Thus, the mixture of HMOs was more effective than prebiotics in altering systemic and gastrointestinal immune cell populations.

G. ALLERGENICITY

As described in Section VI.E, Nowak-Wegrzyn et al. (2019) conducted a cross-over study to evaluate the allergenicity of an LNnT-containing infant formula in 67 infants 2 months to 4 years of age with documented cow milk protein allergy. Although a limited number of adverse events were reported, the LNnT formula met the definition of a hypoallergenic formula as defined by the American Academy of Pediatrics (Nutrition, 2000). These findings indicate that LNnT does not provoke allergic reactions in CMA infants. Additionally, product specifications are in place to control the level of protein derived from the production organisms in the finished ingredient (see Section II.G) and the genetically engineered strains of *E. coli* BL21(DE3) have been safely used in the production of food and pharmaceutical ingredients (see Section VI.A). Moreover, the genes used to engineer the production strains are not derived from major allergens and full-length amino acid alignments of the gene products expressed in the

production strains with 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 7). Thus, although the protein specification does not eliminate the possibility that consumers of Jennewein's LNnT-containing product may be exposed to the protein residues derived from the production organism completely (specification of ≤ 0.01 % protein), allergic reactions resulting from the exposure to theoretically possible protein residues derived from genetically engineered strains of *E. coli* BL21(DE3) in the finished ingredient are not expected.

Table 7. Percent Identity of the Genetic Manipulations in Production Strains with Known Allergens

Gene	Function	Origin of the gene	% Identity*
bbhI	β-*N*-acetylhexosaminidases	*Bifidobacterium bifidum*	26.1
galE	UDP-galactose-4-epimerase	*E. coli* K12	30.7
galF	Regulatory subunit of GalU	*E. coli* K12	None
galK	Galactokinase	*E. coli* K12	None
galM	Galactose mutarotase	*E. coli* K12	26.9
galT	β-1,4-galactosyltransferase	*Aggregatibacter aphrophilus*	24.0
galU	UTP-glucose-1-phosphate uridyltransferase	*E. coli* K12	None
lgtA	β-1,3-N-acetylglucosaminyltransferase	*Neisseria meningitidis*	25.9
lacZ	β-galactosidase	*E. coli* BL21(DE3)	None
lacY	Lactose permease	*E. coli* K12	27.4
nagA	N-acetylglucosamine-6-phosphate deacetylase	*E. coli* BL21(DE3)	30.5
nagB	Glucosamine-6-phosphate deaminase	*E. coli* BL21(DE3)	None
nagE	PTS system N-acetylglucosamine-specific EIICBA component	*E. coli* BL21(DE3)	23.3
pgm	Phosphoglucomutase	*E. coli* K12	29.2
Antibiotic Resistance Genes			
aacC1	Gentamycin 3'-acetyltransferase conferring resistance to gentamycin	*Acinetobacter baumannii AYE*	None
aad1	Aminoglycoside adenyltransferase conferring resistance to streptomycin	*Staphylococcus aureus* strain RIGLD7	None
cat	Chloramphenicol acetyl transferase conferring resistance to chloramphenicol	*Shigella sonnei*	24.3
nptII	Neomycin-phosphotransferase II conferring resistance to kanamycin	TTn5 *E. coli* K12	None
Sh ble	Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	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.

H. REGULATORY APPROVALS AROUND THE WORLD

Two GRAS notices for LNnT products, both submitted by Glycom A/S, have received "no questions" letters from FDA (GRN 547; GRN 659). GRN 547 allows for use of a chemically synthesized LNnT as an ingredient in baked goods and baking mixes, beverages and beverage bases, coffee and tea, dairy product analogs, infant and toddler foods, grain products and pastas, milk (whole and skim), milk products, processed fruits and fruit juices, processed vegetables and vegetable juices, and sugar substitutes at maximum levels ranging from 0.02 to 1.2 g/serving and for use as an ingredient in term infant formula at a maximum level of 0.6 g/L reconstituted formula. GRN 659 allows for use of LNnT produced by fermentation as an ingredient in non-exempt term infant formulas at a maximum use level of 0.6g/L reconstituted formula and in beverages and beverage bases, dairy product analogs, milk (whole and skim), milk products, processed fruits and juices, grain products and pastas, infant foods, and toddler foods (including follow-on formulas) at use levels ranging from 0.02 to 3 g/serving.

LNnT is also approved as a Novel Food in the European Union and the subject of a review by the European Food Safety Authority (Commission Implementing Regulation (EU) 2018/1023; EFSA Panel on Dietetic Products, 2015). During their review, the European Food Safety Authority (EFSA) concluded that LNnT is safe for infants (up to one year of age) when added to infant and follow-on formulae, in combination with 2'-fucosyllactose (2'-FL), at concentrations up to 0.6 g/L of LNnT and up to 1.2 g/L of 2'-FL, at a ratio of 1:2 in the reconstituted formulae; is safe for young children (older than one year of age) when added to follow-on and young-child formulae, at concentrations up to 0.6 g/L of LNnT (alone or in combination with 2'-FL, at concentrations up to 1.2 g/L, at a ratio of 1:2); and is safe when added to other foods at the uses and use levels proposed (0.6-1.2 g/serving depending on food category, 1.5 g/day in supplement form). Additionally, EFSA considered the changes in reticulocytes, platelet counts, hemoglobin levels, and pack cell volume in the high-dose LNnT group (5000 mg/kg body weight/day) and the decrease in the zymogen content in acinar cells in three animals noted in the supporting genotoxicity and subchronic toxicity studies conducted by Coulet et al. (2013) as significant and established their own NOAEL of 2500 mg/kg body weight/day. The OECD-compliant 90-day toxicology study that was conducted with the LNnT product produced by fermentation, summarized in GRN 659 (pg. 33-34), and corroborated the NOAEL established by Coulet et al., 2013) was not reviewed. Importantly, the EDIs of Jennewein's LNnT mimic the levels found in breast milk and are supported by the NOAELs established by Coulet et al. (2013), the unpublished study in summarized in GRN 659, and the European Food Safety Authority.

VII. SUPPORTING DATA AND INFORMATION

A. REFERENCES

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

Alderete, T.L., Autran, C., Brekke, B.E., Knight, R., Bode, L., Goran, M.I., and Fields, D.A. (2015). Associations between human milk oligosaccharides and infant body composition in the first 6 mo of life. Am J Clin Nutr 102, 1381–1388.

Archer, C.T., Kim, J.F., Jeong, H., Park, J.H., Vickers, C.E., Lee, S.Y., and Nielsen, L.K. (2011). The genome sequence of E. coli W (ATCC 9637): comparative genome analysis and an improved genome-scale reconstruction of E. coli. BMC Genomics *12*, 9.

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

Asakuma, S., Urashima, T., Akahori, M., Obayashi, H., Nakamura, T., Kimura, K., Watanabe, Y., Arai, I., and Sanai, Y. (2008). Variation of major neutral oligosaccharides levels in human colostrum. European Journal of Clinical Nutrition *62*, 488–494.

Austin, S., De Castro, C.A., Sprenger, N., Binia, A., Affolter, M., Garcia-Rodenas, C.L., Beauport, L., Tolsa, J.F., and Fischer Fumeaux, C.J. (2019). Human Milk Oligosaccharides in the Milk of Mothers Delivering Term versus Preterm Infants. Nutrients *11*,

Austin, S., De Castro, C., Bénet, T., Hou, Y., Sun, H., Thakkar, S., Vinyes-Pares, G., Zhang, Y., and Wang, P. (2016). Temporal Change of the Content of 10 Oligosaccharides in the Milk of Chinese Urban Mothers. Nutrients *8*, 346.

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

Bao, Y., Chen, C., and Newburg, D.S. (2013). Quantification of neutral human milk oligosaccharides by graphitic carbon high-performance liquid chromatography with tandem mass spectrometry. Analytical Biochemistry *433*, 28–35.

Chart, H., Smith, H.R., La Ragione, R.M., and Woodward, M.J. (2000). An investigation into the pathogenic properties of Escherichia coli strains BLR, BL21, DH5alpha and EQ1. J Appl Microbiol *89*, 1048–1058.

Chaturvedi, P., Warren, C.D., Altaye, M., Morrow, A.L., Ruiz-Palacios, G., Pickering, L.K., and Newburg, D.S. (2001). Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation. Glycobiology *11*, 365–372.

Chaturvedi, P., Warren, C.D., Ruiz-Palacios, G.M., Pickering, L.K., and Newburg, D.S. (1997). Milk oligosaccharide profiles by reversed-phase HPLC of their perbenzoylated derivatives. Anal Biochem *251*, 89–97.

Commission Implementing Regulation (EU) 2018/1023 of 23 July 2018 correcting Implementing Regulation (EU) 2017/2470 establishing the Union list of novel foods. L 187/1, 24.7.2018.

Committee on Nutrition. (2000). Hypoallergenic Infant Formulas. Pediatrics *106*, 346–349.

Comstock, S.S., Li, M., Wang, M., Monaco, M.H., Kuhlenschmidt, T.B., Kuhlenschmidt, M.S., and Donovan, S.M. (2017). Dietary Human Milk Oligosaccharides but Not Prebiotic Oligosaccharides Increase Circulating Natural Killer Cell and Mesenteric Lymph Node Memory T Cell Populations in Noninfected and Rotavirus-Infected Neonatal Piglets. J Nutr *147*, 1041–1047.

Coppa, G.V., Pierani, P., Zampini, L., Carloni, I., Carlucci, A., and Gabrielli, O. (1999). Oligosaccharides in human milk during different phases of lactation. Acta Paediatr Suppl *88*, 89–94.

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

Daegelen, P., Studier, F.W., Lenski, R.E., Cure, S., and Kim, J.F. (2009). Tracing ancestors and relatives of Escherichia coli B, and the derivation of B strains REL606 and BL21(DE3). J Mol Biol *394*, 634–643.

Davies, P.S., Wells, J.C., and Lucas, A. (1994). Adjusting milk intake for body size in early infancy. Early Hum Dev *36*, 61–67.

Dotz, V., Rudloff, S., Blank, D., Lochnit, G., Geyer, R., and Kunz, C. (2014). 13C-labeled oligosaccharides in breastfed infants' urine: individual-, structure- and time-dependent differences in the excretion. Glycobiology *24*, 185–194.

EFSA Panel on Dietetic Products, Nutrition and Allergies. (2015). Safety of lacto-N-neotetraose as a novel food ingredient pursuant to Regulation (EC) No 258/97. EFSA Journal *13*, 4183.

Elison, E., Vigsnaes, L.K., Rindom Krogsgaard, L., Rasmussen, J., Sørensen, N., McConnell, B., Hennet, T., Sommer, M.O., and Bytzer, P. (2016). Oral supplementation of healthy adults with 2'-O-fucosyllactose and lacto-N-neotetraose is well tolerated and shifts the intestinal microbiota. Br J Nutr *116*, 1356–1368.

Ellis, H.M., Yu, D., DiTizio, T., and Court, D.L. (2001). High efficiency mutagenesis, repair, and engineering of chromosomal DNA using single-stranded oligonucleotides. Proc Natl Acad Sci U S A *98*, 6742–6746.

Engfer, M.B., Stahl, B., Finke, B., Sawatzki, G., and Daniel, H. (2000). Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr *71*, 1589–1596.

Erney, R.M., Malone, W.T., Skelding, M.B., Marcon, A.A., Kleman–Leyer, K.M., O'Ryan, M.L., Ruiz–Palacios, G., Hilty, M.D., Pickering, L.K., and Prieto, P.A. (2000). Variability of human milk neutral oligosaccharides in a diverse population. Journal of pediatric gastroenterology and nutrition *30*, 181–192.

Gabrielli, O., Zampini, L., Galeazzi, T., Padella, L., Santoro, L., Peila, C., Giuliani, F., Bertino, E., Fabris, C., and Coppa, G.V. (2011). Preterm milk oligosaccharides during the first month of lactation. Pediatrics *128*, e1520–31.

Galeotti, F., Coppa, G.V., Zampini, L., Maccari, F., Galeazzi, T., Padella, L., Santoro, L., Gabrielli, O., and Volpi, N. (2012). On-line high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry profiling of human milk oligosaccharides derivatized with 2-aminoacridone. Anal Biochem *430*, 97–104.

Gnoth, M.J., Kunz, C., Kinne-Saffran, E., and Rudloff, S. (2000). Human milk oligosaccharides are minimally digested in vitro. J Nutr *130*, 3014–3020.

Goehring, K.C., Kennedy, A.D., Prieto, P.A., and Buck, R.H. (2014). Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PLoS One *9*, e101692.

Gridneva, Z., Rea, A., Tie, W.J., Lai, C.T., Kugananthan, S., Ward, L.C., Murray, K., Hartmann, P.E., and Geddes, D.T. (2019). Carbohydrates in Human Milk and Body Composition of Term Infants during the First 12 Months of Lactation. Nutrients *11*, 1472.

GRN 233. (2002). Combination of galacto-oligosaccharides and polydextrose. Mead Johnson & Company. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=233&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 285. (2009). Galacto-oligosaccharides. GTC Nutrition. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=285&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 286. (2009). Galacto-oligosaccharides. GTC Nutrition. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=286&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 334. (2010). Galacto-oligosaccharides. Yakult Pharmaceutical Industry Co., Ltd. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=334&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 392 (2012). Oligofructose. Pfizer Nutrition. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=392&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligofructose)

GRN 477. (2014). Long-chain inulin. Danone Trading B.V. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=477&sort=GRN_No&order=DESC&startrow=1&type=basic&search=inulin)

GRN 484. (2014). Galacto-oligosaccharides. Clasado, Inc. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=484&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 485 (2013). Beta-galactosidase enzyme preparation. Clasado, Inc. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=485&sort=GRN_No&order=DESC&startrow=1&type=basic&search=485)

GRN 489. (2014). Galacto-oligosaccharides. International Dairy Ingredients, Inc. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=489&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 495. (2014). Galacto-oligosaccharides. Clasado, Inc. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=495&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 518. (2014). Galacto-oligosaccharides. New Francisco Biotechnology Corporation. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=518&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 537. (2015) Short-chain fructo-oligosaccharides. Ingredion Incorporated. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=537&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 547. (2014). Lacto-N-neotetraose. Glycom. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=547&sort=GRN_No&order=DESC&startrow=1&type=basic&search=547)

GRN 569. (2015). Galacto-oligosaccharides. New Francisco Biotechnology Corporation. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=569&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 571. (2015). 2'-Fucosyllactose. Jennewein Biotechnologie. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=571&sort=GRN_No&order=DESC&startrow=1&type=basic&search=571)

GRN 576. (2015). Oligofructose and inulin. Nutricia North America, Inc. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=576&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligofructose)

GRN 620. (2016). Galacto-oligosaccharides. Nestle Nutrition. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=620&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 659 (2016). Lacto-N-neotetraose. Glycom. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=659&sort=GRN_No&order=DESC&startrow=1&type=basic&search=659)

GRN 721. (2017). Galacto-oligosaccharides. Vitalus Nutrition Inc. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=721&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 729. (2018). Galacto-oligosaccharides. Neo Cremar, Co., Ltd. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=729&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

GRN 797. (2018). Galacto-oligosaccharides. New Francisco (Yunfu City) Biotechnology Corporation. (https://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&id=797&sort=GRN_No&order=DESC&startrow=1&type=basic&search=oligosaccharide)

Harper, M.S., Carpenter, C., Klocke, D.J., Carlson, G., Davis, T., and Delaney, B. (2011). E. coli Lipopolysaccharide: acute oral toxicity study in mice. Food Chem Toxicol *49*, 1770–1772.

Hester, S.N., Hustead, D.S., Mackey, A.D., Singhal, A., and Marriage, B.J. (2012). Is the macronutrient intake of formula-fed infants greater than breast-fed infants in early infancy. J Nutr Metab *2012*, 891201.

Hoess, R.H., and Abremski, K. (1990). The Cre-lox recombination system. In Nucleic Acids and Molecular Biology 4, Springer), pp. 99–109.

Jeong, H., Barbe, V., Lee, C.H., Vallenet, D., Yu, D.S., Choi, S.-H., Couloux, A., Lee, S.-W., Yoon, S.H., Cattolico, L., Hur, C.-G., Park, H.-S., Ségurens, B., Kim, S.C., Oh, T.K., Lenski, R.E., Studier, F.W., Daegelen, P., and Kim, J.F. (2009). Genome Sequences of Escherichia coli B strains REL606 and BL21(DE3). Journal of Molecular Biology *394*, 644–652.

Jost, T., Lacroix, C., Braegger, C., and Chassard, C. (2015). Impact of human milk bacteria and oligosaccharides on neonatal gut microbiota establishment and gut health. Nutr Rev *73*, 426–437.

Kuntz, S., Kunz, C., Borsch, C., Vazquez, E., Buck, R., Reutzel, M., Eckert, G.P., and Rudloff, S. (2019). Metabolic Fate and Distribution of 2´-Fucosyllactose: Direct Influence on Gut Microbial Activity but not on Brain. Mol Nutr Food Res e1900035.

Kunz, C., Rodrigues-Palmero, M., Koletzko, B., and Jensen, R. (1999). Nutritional and Biochemical Properties of Human Milk, Part 1. Clinical Aspects of Human Milk and Lactation 26, 307–333.

Lambert, J.M., Bongers, R.S., and Kleerebezem, M. (2007). Cre-lox-based system for multiple gene deletions and selectable-marker removal in Lactobacillus plantarum. Appl Environ Microbiol 73, 1126–1135.

Lampe, D.J., Akerley, B.J., Rubin, E.J., Mekalanos, J.J., and Robertson, H.M. (1999). Hyperactive transposase mutants of the Himar1 mariner transposon. Proc Natl Acad Sci U S A *96*, 11428–11433.

Larsson, M.W., Lind, M.V., Laursen, R.P., Yonemitsu, C., Larnkjær, A., Mølgaard, C., Michaelsen, K.F., and Bode, L. (2019). Human Milk Oligosaccharide Composition Is Associated With Excessive Weight Gain During Exclusive Breastfeeding-An Explorative Study. Front Pediatr 7, 297.

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

Milani, C., Duranti, S., Bottacini, F., Casey, E., Turroni, F., Mahony, J., Belzer, C., Delgado Palacio, S., Arboleya Montes, S., Mancabelli, L., Lugli, G.A., Rodriguez, J.M., Bode, L., de Vos, W., Gueimonde, M., Margolles, A., van Sinderen, D., and Ventura, M. (2017). The First Microbial Colonizers of the Human Gut: Composition, Activities, and Health Implications of the Infant Gut Microbiota. Microbiol Mol Biol Rev *81*,

National Institutes of Health. (2019). NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines). Available at https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.html, December 20, 2019.

Nakhla, T., Fu, D., Zopf, D., Brodsky, N.L., and Hurt, H. (1999). Neutral oligosaccharide content of preterm human milk. Br J Nutr *82*, 361–367.

Nowak-Wegrzyn, Czerkies, Reyes, Collins, and Heine (2019). Confirmed Hypoallergenicity of a Novel Whey-Based Extensively Hydrolyzed Infant Formula Containing Two Human Milk Oligosaccharides. Nutrients *11*, 1447.

Obermeier, S., Rudloff, S., Pohlentz, G., Lentze, M.J., and Kunz, C. (1999). Secretion of 13C-labelled oligosaccharides into human milk and infant's urine after an oral 13C-galactose load. Isotopes in environmental and health studies *35*, 119–125.

Prieto, P.A. (2005). In Vitroand Clinical Experiences with a Human Milk Oligosaccharide, Lacto-N-neoTetraose, and Fructooligosaccharides.

Puccio, G., Alliet, P., Cajozzo, C., Janssens, E., Corsello, G., Sprenger, N., Wernimont, S., Egli, D., Gosoniu, L., and Steenhout, P. (2017). Effects of Infant Formula With Human Milk Oligosaccharides on Growth and Morbidity: A Randomized Multicenter Trial. J Pediatr Gastroenterol Nutr *64*, 624–631.

Rudloff, S., and Kunz, C. (2012). Milk oligosaccharides and metabolism in infants. Adv Nutr *3*, 398S–405S.

Rudloff, S., Obermeier, S., Borsch, C., Pohlentz, G., Hartmann, R., Brösicke, H., Lentze, M.J., and Kunz, C. (2006). Incorporation of orally applied (13)C-galactose into milk lactose and oligosaccharides. Glycobiology *16*, 477–487.

Rudloff, S., Pohlentz, G., Diekmann, L., Egge, H., and Kunz, C. (1996). Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula. Acta Paediatrica *85*, 598–603.

Rudloff, S., Pohlentz, G., Borsch, C., Lentze, M.J., and Kunz, C. (2012). Urinary excretion of in vivo 13C-labelled milk oligosaccharides in breastfed infants. British Journal of Nutrition *107*, 957–963.

Ruhaak, L.R., Stroble, C., Underwood, M.A., and Lebrilla, C.B. (2014). Detection of milk oligosaccharides in plasma of infants. Anal Bioanal Chem *406*, 5775–5784.

Santos-Fandila, A., Zafra-Gómez, A., Vazquez, E., Navalón, A., Rueda, R., and Ramírez, M. (2014). Ultra high performance liquid chromatography–tandem mass spectrometry method for the determination of soluble milk glycans in rat serum. Talanta *118*, 137–146.

Section on Breastfeeding, American Academy of Pediatrics (2012). Breastfeeding and the use of human milk. Pediatrics *129*, e827–41.

Smilowitz, J.T., Lebrilla, C.B., Mills, D.A., German, J.B., and Freeman, S.L. (2014). Breast milk oligosaccharides: structure-function relationships in the neonate. Annu Rev Nutr *34*, 143–169.

Sprenger, N., Lee, L.Y., De Castro, C.A., Steenhout, P., and Thakkar, S.K. (2017). Longitudinal change of selected human milk oligosaccharides and association to infants' growth, an observatory, single center, longitudinal cohort study. PLoS One *12*, e0171814.

Studier, F.W., Daegelen, P., Lenski, R.E., Maslov, S., and Kim, J.F. (2009). Understanding the differences between genome sequences of Escherichia coli B strains REL606 and BL21(DE3) and comparison of the E. coli B and K-12 genomes. J Mol Biol *394*, 653–680.

Sumiyoshi, W., Urashima, T., Nakamura, T., Arai, I., Saito, T., Tsumura, N., Wang, B., Brand-Miller, J., Watanabe, Y., and Kimura, K. (2003). Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. Br J Nutr *89*, 61–69.

Thurl, S., Henker, J., Siegel, M., Tovar, K., and Sawatzki, G. (1997). Detection of four human milk groups with respect to Lewis blood group dependent oligosaccharides. Glycoconj J *14*, 795–799.

Thurl, S., Munzert, M., Henker, J., Boehm, G., Müller-Werner, B., Jelinek, J., and Stahl, B. (2010). Variation of human milk oligosaccharides in relation to milk groups and lactational periods. British Journal of Nutrition *104*, 1261–1271.

Vandenplas, Y., Berger, B., Carnielli, V.P., Ksiazyk, J., Lagström, H., Sanchez Luna, M., Migacheva, N., Mosselmans, J.M., Picaud, J.C., Possner, M., Singhal, A., and Wabitsch, M. (2018). Human Milk Oligosaccharides: 2'-Fucosyllactose (2'-FL) and Lacto-N-Neotetraose (LNnT) in Infant Formula. Nutrients *10*, 1161.

Van Niekerk, E., Autran, C.A., Nel, D.G., Kirsten, G.F., Blaauw, R., and Bode, L. (2014). Human Milk Oligosaccharides Differ between HIV-Infected and HIV-Uninfected Mothers and Are Related to Necrotizing Enterocolitis Incidence in Their Preterm Very-Low-Birth-Weight Infants. The Journal of Nutrition *144*, 1227–1233.

Vazquez, E., Santos-Fandila, A., Buck, R., Rueda, R., and Ramirez, M. (2017). Major human milk oligosaccharides are absorbed into the systemic circulation after oral administration in rats. British Journal of Nutrition *117*, 237–247.

Williams, J.E., Price, W.J., Shafii, B., Yahvah, K.M., Bode, L., McGuire, M.A., and McGuire, M.K. (2017). Relationships among microbial communities, maternal cells, oligosaccharides, and macronutrients in human milk. Journal of Human Lactation *33*, 540–551.

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-neo*tetraose (LNnT) as an ingredient for the intended uses in infant formula, and conventional foods and beverages. LNnT 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).

Jennewein Biotechnologie is proposing to market LNnT for the intended uses specified above. The proposed use of LNnT as an ingredient for the intended uses in infant formula, and conventional foods and beverages has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. LNnT is one of the most abundant (0.1-0.6%) oligosaccharides in human milk. Human milk oligosaccharides, including LNnT, 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.

2. The LNnT that is the subject of this GRAS determination is a spray-dried, powdered food ingredient that contains not less than 92% LNnT and is manufactured by fermentation using two genetically engineered strains of *Escherichia coli* BL21(DE3). By-products include lactose, the intermediate lactose-N-triaose 2 (LNT II), and *para*-lacto-*N-neo*hexaose (pLNnH), which are also human milk oligosaccharides and their presence in the finished ingredient is not unexpected.

3. The subject of this GRAS Notification is structurally identical to human milk LNnT and is chemically equivalent to the subjects of GRAS Notifications (GRNs) 547 and 659, both of which received “no questions” letters from FDA.

4. Jennewein is a Food Facility registered with FDA.

5. LNnT 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.

6. LNnT is manufactured with the help of the two genetically engineered production strains of *Escherichia coli* BL21(DE3). Because *E. coli* BL21(DE3) does not

possess the components required for *E. coli* pathogenicity, the production strains are non-pathogenic.

7. All raw materials, processing aids, and food contact substances are GRAS.

8. 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 endotoxin, ensuring a consistent, food-grade finished ingredient.

9. Because the subject of this GRAS notification is structurally identical to the LNnT present in human breast milk and subjects of GRN 547 and 659, and chemically equivalent to the subjects of GRN 547 and 659, the genotoxicology and 90-day toxicology studies published by Coulet et al. (2013), which support the safe use of the subject of GRN 547, are the pivotal studies that support the safe use of LNnT in infant formulas, selected conventional foods and beverages.

    a. LNnT is not genotoxic.

    b. The NOAEL for orally administered LNnT reported by Coulet et al. (2013) is at least 5000 mg/kg/day.

    c. Additional genotoxicology and 90-day toxicology studies summarized in GRN 659 corroborate the results published by Coulet et al. (2013).

10. Clinical studies have reported that the ingestion of 1 g/L and 20 g/d of LNnT is well-tolerated in infants and adults, respectively.

11. The intended uses and corresponding use levels will result in a mean and 90th percentile estimated daily intakes (EDIs) of 510 and 730 mg/day (83.2 and 133.9 mg/kg bw/day, respectively) in 0 to 6-month old infants consuming LNnT-containing formulas, mean and 90th percentile EDIs of 420 and 660 mg/day in 7 to 12 month old infants, and mean and 90th percentile EDIs of 304 and 646 mg/day (8.1 and 16.8 mg/kg bw/day, respectively) from selected conventional foods and beverages.

12. The EDI is substantially below the safe level of intake for LNnT determined in the toxicology and corroborating clinical studies, thereby establishing the safety of LNnT ingestion from the intended uses and use levels.

Therefore, LNnT is safe and GRAS at the proposed levels of addition to the intended infant and follow-on formula, products for children, and conventional foods and beverages. Lacto-*N-neo*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: February 6, 2020

A. Wallace Hayes, PhD, DABT, FATS, ERT
GRAS Expert Panel Member
Harvard School of Public Health

Signature: [redacted]

Date: February 6, 2020

Thomas E. Sox, PhD, JD
GRAS Expert Panel Member
Principal, Pondview Consulting LLC

Signature: [redacted]

Date: February 6, 2020

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel
Spherix Consulting Group, Inc.

Signature: [redacted]

Date: February 6, 2020

From:	kbrailer@spherixgroup.com
To:	Morissette, Rachel; "Dietrich Conze"
Cc:	"Claire Kruger"
Subject:	RE: GRN 000919 questions
Date:	Friday, July 17, 2020 2:40:13 PM
Attachments:	image001.png Response to FDA Questions on GRN919 7-17-20.pdf

Dear Rachel,

Attached please find our response to your questions regarding GRN 000919.

Best regards,

Kathy Brailer
Director of Administrative Services
Spherix Consulting Group, Inc.
11821 Parklawn Drive, Suite 310
Rockville, MD 20852
+1-301-557-0375
kbrailer@spherixgroup.com
www.spherixgroup.com

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Thursday, June 25, 2020 12:24 PM
To: Dietrich Conze <dconze@spherixgroup.com>
Cc: Kathy Brailer <kbrailer@spherixgroup.com>; Claire Kruger <ckruger@spherixgroup.com>
Subject: RE: GRN 000919 questions

That will be fine.

Best,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

FDA U.S. FOOD & DRUG ADMINISTRATION

From: Dietrich Conze <dconze@spherixgroup.com>

Sent: Thursday, June 25, 2020 12:13 PM
To: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Cc: Kathy Brailer <kbrailer@spherixgroup.com>; Claire Kruger <ckruger@spherixgroup.com>
Subject: Re: GRN 000919 questions

Great. Would it be possible to send you our responses on July 20th? That would be a total of 17 days, not including July 3rd.
Dietz

> On Jun 25, 2020, at 11:30 AM, Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:
>
> Sure, how much time are you thinking?
>
> *Rachel*
> ---
> **Rachel Morissette, Ph.D.**
> *Regulatory Review Scientist*
>
> **Division of Food Ingredients**
> **Office of Food Additive Safety**
> **Center for Food Safety and Applied Nutrition**
> **U.S. Food and Drug Administration**
> rachel.morissette@fda.hhs.gov
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>
> **From:** Dietrich Conze <dconze@spherixgroup.com>
> **Sent:** Thursday, June 25, 2020 11:29 AM
> **To:** Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
> **Cc:** Kathy Brailer <kbrailer@spherixgroup.com>; Claire Kruger <ckruger@spherixgroup.com>
> **Subject:** Re: GRN 000919 questions
>
> Hi Rachel,
> We've reviewed the questions. Due to the approaching holiday, vacation schedules, a scheduled surgery, and time needed to coordinate with our client, we are wondering if it would be possible to obtain an extension to July 20th to address questions 1-10.
>
> Regards.
> Dietz
>
> Dietrich Conze, PhD
> Managing Partner
> Spherix Consulting Group

11821 Parklawn Drive, Suite 310
Rockville, MD 20852

Tel: 240-367-6089
Fax: 301-230-2188
dconze@spherixgroup.com

On Jun 24, 2020, at 3:06 PM, Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:

Dear Dr. Conze,

Please see attached our questions for GRN 000919. Please let me know if you have any questions at this time.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist

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

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<2020-06-24 GRN 000919 Questions for Notifier.pdf>

Spherix Consulting Group

July 17, 2020

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

RE: Questions Regarding GRN 000919

Dear Dr. Morissette:

In response to your email of June 24, 2020, following are our responses to your request for additional information regarding GRN 000919. FDA's questions are italicized and our responses are in plain text.

Regulatory:

1. *Please note that although the U.S. does not have a definition for "toddler formula", we recognize it as formula intended for >12 months of age. However, if it is intended for infants <12 months of age (e.g., 9-18 months), then those products must comply with the infant formula regulations under Section 412 of the Federal Food, Drug, and Cosmetic Act as the intended population includes infants <12 months of age.*

   Thank you for the guidance. Please note that Jennewein manufactures ingredients intended for infant formulas and conventional foods, not infant formulas. Per Section 412 of the Federal Food, Drug, and Cosmetic Act, all infant formula manufacturers must notify the U.S. FDA at least 90 days before marketing their infant formula, specify the quantitative formulation of each form of the infant formula, and provide assurance that the infant formula will not be marketed unless the formula meets the requirements of Section 412 of the Federal Food, Drug, and Cosmetic Act. Therefore, as an ingredient manufacturer, it is not the responsibility of Jennewein to ensure that its products comply with infant formula regulations.

2. *We do not have a regulatory definition for "follow-on formula." Please clarify what is meant by this term.*

   Follow-on formula/toddler formula is defined by the World Health Organization as "food intended for use as a liquid part of the weaning diet for the infant from the 6th month on and for young children" (World Health Organization, 2001).

11821 Parklawn Drive, Suite 310
Rockville, MD 20852
301-557-0375; info@spherixgroup.com
http://spherixgroup.html

3. Please provide a statement that Jennewein concludes that the intended use of LNnT is GRAS.

Based on the available data, there is reasonable certainty that the use of Jennewein's LNnT per the intended uses and use levels is of no harm to consumers. Jennewein therefore concludes that the intended use of LNnT as an ingredient in conventional foods and beverages, as well as non-exempt, term infant formula at the intended use levels is GRAS.

Chemistry:

4. Please clarify the intended source of the infant formula protein base (e.g., milk, soy, whey) into which the LNnT would be added.

LNnT will be added to milk- and whey protein-based infant formulas.

5. Please state that all analytical methods used for the batch analyses have been validated and are fit for purpose.

All analytical methods have been validated and are fit-for-purpose.

6. Please include a statement that all steps in the manufacturing process follow current good manufacturing practices.

On page 10 of the Notice, please replace the following statement: "Lacto-*N*-*neo*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 an FDA-registered Food Facility (Registration # 1303109037512" with "Lacto-*N*-*neo*tetraose production occurs at the Jennewein Biotechnologie GmbH production facility in Maarweg 32, 53619 Rheinbreitbach, Germany, according to Good Manufacturing Practices in a Food Safety System Certification (FSSC) 22000- and ISO 9001:2015-compliant, and an FDA-registered Food Facility (Registration # 1303109037512)."

Microbiology:

7. On page 7 of the notice, Jennewein states the following:

"Although PS-LNnT-JBT possesses the zeocin (Sh ble) and chloramphenicol (cat) resistance genes that were used for integrant selection, no plasmids or other episomal vectors remain in the genome, thus conferring a high degree of genetic stability."

A similar statement is provided for the DS-LNnT-JBT clone.

Please provide the number of generations that were evaluated for genetic stability of the two clones, PS-LNnT-JBT and DS-LNnT-JBT, used to produce LNnT.

The genetic stability of the production strain PS-LNnT-JBT has been evaluated over approximately 20 cell generations. The genetic stability of the degradation strain DS-LNnT-JBT has been evaluated over approximately five generations.

8. *Please list all antibiotic genes that are still present in the two clones, PS-LNnT-JBT and DS-LNnT-JBT, and confirm that they are located on the genome, which was confirmed by polymerase chain reaction (PCR) using oligonucleotides specific to the coding sequence and genomic DNA.*

The PS-L*N*nT-JBT genome possesses the zeocin (*Sh ble*) and chloramphenicol (*cat*) resistance genes, which was confirmed by polymerase chain reaction (PCR) using oligonucleotides specific to the coding sequence and genomic DNA.

The DS-L*N*nT-JBT genome contains the streptomycin (*aad1*), zeocin (*Sh ble*), kanamycin (*nptII*), and gentamycin (*aacC1*) resistance genes, which was confirmed by PCR using oligonucleotides specific to the coding sequence and genomic DNA.

9. *On page 8 of the notice, Jennewein states the following:*

"...all plasmids and other episomal vectors were removed from genome."

Please confirm that the clones, PS-LNnT-JBT and DS-LNnT-JBT, do not carry any additional plasmids or describe the identity and genes encoded on any additional plasmids that would be present in the production clones.

Both PS-LNnT-JBT and DS-LNnT-JBT are devoid of additional plasmids, which has been confirmed by growth at 42°C, ampicillin sensitivity, failure to amplify plasmid specific DNA, and whole genome sequencing.

10. *If the two clones, PS-LNnT-JBT and DS-LNnT-JBT, were deposited, please provide the genebank deposit information.*

The degradation strain DS-LNnT-JBT has been deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) with the No. DSM 33488. The deposition of the production strain PS-LNnT-JBT is in process.

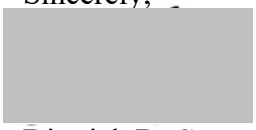
Toxicology:

11. Please clarify if LNnT is intended to be formulated with other human milk oligosaccharides (HMOs), such as 2'-fucosyllactose, lacto-N-tetraose, 3'-sialyllactose (SL), 6'-SL, and/or other indigestible oligosaccharides on the market. Please indicate the target level of total HMOs in the infant formula and a rationale why the level would not be of concern (i.e., causing adverse gastrointestinal effects).

LNT is intended to be added to infant formula to produce an infant formula that is compositionally representative of human breast milk. LNnT may therefore be used by an infant formula manufacturer either alone or in combination with other human milk oligosaccharides (HMOs) to match the levels that occur in breast milk. Importantly, Jennewein manufactures infant formula ingredients, not infant formula. Per Section 412(d)(1) of the Federal Food, Drug, and Cosmetic Act (FFDCA), manufacturers of new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula and their Infant Formula Notification must include a description of any reformulation of the formula or change in processing of the infant formula and evidence showing that a particular oligosaccharide Combination (*e.g.*, use of LNnT with an indigestible oligosaccharide such as GOS) would be well tolerated. Therefore, it is not the responsibility of Jennewein to determine the target level of total HMOs in the infant formula or provide the rationale for why the level would not be of concern.

Should you need additional information, please feel free to contact me at 240-367-6089 or dconze@spherixgroup.com.

Sincerely,



Dietrich B. Conze, Ph.D.
Managing Partner

References

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

Spherix Consulting Group

July 17, 2020

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

RE: Questions Regarding GRN 000919

Dear Dr. Morissette:

In response to your email of June 24, 2020, following are our responses to your request for additional information regarding GRN 000919. FDA's questions are italicized and our responses are in plain text.

Regulatory:

1. *Please note that although the U.S. does not have a definition for "toddler formula", we recognize it as formula intended for >12 months of age. However, if it is intended for infants <12 months of age (e.g., 9-18 months), then those products must comply with the infant formula regulations under Section 412 of the Federal Food, Drug, and Cosmetic Act as the intended population includes infants <12 months of age.*

   Thank you for the guidance. Please note that Jennewein manufactures ingredients intended for infant formulas and conventional foods, not infant formulas. Per Section 412 of the Federal Food, Drug, and Cosmetic Act, all infant formula manufacturers must notify the U.S. FDA at least 90 days before marketing their infant formula, specify the quantitative formulation of each form of the infant formula, and provide assurance that the infant formula will not be marketed unless the formula meets the requirements of Section 412 of the Federal Food, Drug, and Cosmetic Act. Therefore, as an ingredient manufacturer, it is not the responsibility of Jennewein to ensure that its products comply with infant formula regulations.

2. *We do not have a regulatory definition for "follow-on formula." Please clarify what is meant by this term.*

   Follow-on formula/toddler formula is defined by the World Health Organization as "food intended for use as a liquid part of the weaning diet for the infant from the 6th month on and for young children" (World Health Organization, 2001).

11821 Parklawn Drive, Suite 310
Rockville, MD 20852
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3. Please provide a statement that Jennewein concludes that the intended use of LNnT is GRAS.

Based on the available data, there is reasonable certainty that the use of Jennewein's LNnT per the intended uses and use levels is of no harm to consumers. Jennewein therefore concludes that the intended use of LNnT as an ingredient in conventional foods and beverages, as well as non-exempt, term infant formula at the intended use levels is GRAS.

Chemistry:

4. Please clarify the intended source of the infant formula protein base (e.g., milk, soy, whey) into which the LNnT would be added.

LNnT will be added to milk- and whey protein-based infant formulas.

5. Please state that all analytical methods used for the batch analyses have been validated and are fit for purpose.

All analytical methods have been validated and are fit-for-purpose.

6. Please include a statement that all steps in the manufacturing process follow current good manufacturing practices.

On page 10 of the Notice, please replace the following statement: "Lacto-*N*-*neo*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 an FDA-registered Food Facility (Registration # 1303109037512" with "Lacto-*N*-*neo*tetraose production occurs at the Jennewein Biotechnologie GmbH production facility in Maarweg 32, 53619 Rheinbreitbach, Germany, according to Good Manufacturing Practices in a Food Safety System Certification (FSSC) 22000- and ISO 9001:2015-compliant, and an FDA-registered Food Facility (Registration # 1303109037512)."

Microbiology:

7. On page 7 of the notice, Jennewein states the following:

"Although PS-LNnT-JBT possesses the zeocin (Sh ble) and chloramphenicol (cat) resistance genes that were used for integrant selection, no plasmids or other episomal vectors remain in the genome, thus conferring a high degree of genetic stability."

A similar statement is provided for the DS-LNnT-JBT clone.

Please provide the number of generations that were evaluated for genetic stability of the two clones, PS-LNnT-JBT and DS-LNnT-JBT, used to produce LNnT.

The genetic stability of the production strain PS-LNnT-JBT has been evaluated over approximately 20 cell generations. The genetic stability of the degradation strain DS-LNnT-JBT has been evaluated over approximately five generations.

8. *Please list all antibiotic genes that are still present in the two clones, PS-LNnT-JBT and DS-LNnT-JBT, and confirm that they are located on the genome, which was confirmed by polymerase chain reaction (PCR) using oligonucleotides specific to the coding sequence and genomic DNA.*

The PS-L*N*nT-JBT genome possesses the zeocin (*Sh ble*) and chloramphenicol (*cat*) resistance genes, which was confirmed by polymerase chain reaction (PCR) using oligonucleotides specific to the coding sequence and genomic DNA.

The DS-L*N*nT-JBT genome contains the streptomycin (*aad1*), zeocin (*Sh ble*), kanamycin (*nptII*), and gentamycin (*aacC1*) resistance genes, which was confirmed by PCR using oligonucleotides specific to the coding sequence and genomic DNA.

9. *On page 8 of the notice, Jennewein states the following:*

"...all plasmids and other episomal vectors were removed from genome."

Please confirm that the clones, PS-LNnT-JBT and DS-LNnT-JBT, do not carry any additional plasmids or describe the identity and genes encoded on any additional plasmids that would be present in the production clones.

Both PS-LNnT-JBT and DS-LNnT-JBT are devoid of additional plasmids, which has been confirmed by growth at 42°C, ampicillin sensitivity, failure to amplify plasmid specific DNA, and whole genome sequencing.

10. *If the two clones, PS-LNnT-JBT and DS-LNnT-JBT, were deposited, please provide the genebank deposit information.*

The degradation strain DS-LNnT-JBT has been deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) with the No. DSM 33488. The deposition of the production strain PS-LNnT-JBT is in process.

Toxicology:

11. Please clarify if LNnT is intended to be formulated with other human milk oligosaccharides (HMOs), such as 2'-fucosyllactose, lacto-N-tetraose, 3'-sialyllactose (SL), 6'-SL, and/or other indigestible oligosaccharides on the market. Please indicate the target level of total HMOs in the infant formula and a rationale why the level would not be of concern (i.e., causing adverse gastrointestinal effects).

LNT is intended to be added to infant formula to produce an infant formula that is compositionally representative of human breast milk. LNnT may therefore be used by an infant formula manufacturer either alone or in combination with other human milk oligosaccharides (HMOs) to match the levels that occur in breast milk. Importantly, Jennewein manufactures infant formula ingredients, not infant formula. Per Section 412(d)(1) of the Federal Food, Drug, and Cosmetic Act (FFDCA), manufacturers of new infant formula must notify the U.S. FDA at least 90 days before marketing their infant formula and their Infant Formula Notification must include a description of any reformulation of the formula or change in processing of the infant formula and evidence showing that a particular oligosaccharide Combination (*e.g.*, use of LNnT with an indigestible oligosaccharide such as GOS) would be well tolerated. Therefore, it is not the responsibility of Jennewein to determine the target level of total HMOs in the infant formula or provide the rationale for why the level would not be of concern.

Should you need additional information, please feel free to contact me at 240-367-6089 or dconze@spherixgroup.com.

Sincerely,

Dietrich B. Conze, Ph.D.
Managing Partner

References

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