Spherix Consulting Group

March 19, 2020

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

Dear Dr. Morissette:

It is our opinion that the enclosed GRAS Determination for the Use of 3'-Sialyllactose (3'-SL) in Non-Exempt Term Infant Formula constitutes a new notification. Although 3'-SL is the subject of GRAS Notices 766 and 880, the subject of the enclosed GRAS Notice is produced using a novel production process and the intended use levels are greater than those that have been determined GRAS.

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

Sincerely,

Dietrich B. Conze, Ph.D.
Managing Partner

Enclosure:

CD containing Form 3667, cover letter, GRAS Determination for the Use of 3'-Sialyllactose (3'-SL) in Non-Exempt Term Infant Formula, and all references

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GRAS Determination for the Use of 3'-Sialyllactose Sodium Salt in Non-Exempt Term Infant Formula

Prepared for:

Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

Prepared by:

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March 19, 2020

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LIST OF ABBREVIATIONS

2'-FL: 2'-Fucosyllactose

3-FL: 3-Fucosyllactose

3'-SL: 3'-Sialyllactose

6'-SL: 6'-Sialyllactose

Alb: Albumin

ALT: Alanine aminotransferase

araA: arabinose isomerase

BMI: Body mass index

BW: Body weight

CBPI: Cytokinesis-block proliferation index

CFR: United States Code of Federal Regulations

CFU: Colony forming units

CHO: Chinese hamster ovary cells

CI: Confidence interval

COSY: Correlation spectroscopy

DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen

DW: Dry weight

EDI: Estimated daily intake

EFSA: European Food Safety Authority

EU: Endotoxin unit

F6PPK: Fructose-6-phosphate phosphoketolase

FCC: Food Chemicals Codex

FDA: United States Food and Drug Administration

FFDCA: Federal Food, Drug, and Cosmetic Act

FOIA: Freedom of information Act

FOS: Fructooligosaccharides

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

Fru-6-P: Fructose-6-phosphate

FSSC: Food Safety System Certification

FUT: Fucosyltransferase

GI: Gastrointestinal

Glc-1-P: Glucose-1-phosphate

Glc-6-P: Glucose-6-phosphate

Gln-1-P: Glucosamine-1-phosphate

Gln-6-P: Glucosamine-6-phosphate

Glob: Gobulin

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

GMO: Genetically modified organism

GMP: Good manufacturing practices

GOS: Galactooligosaccharides

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

HCD: Historical control data

HDL-C: High-density lipoprotein cholesterol

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

HMO: Human milk oligosaccharides

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

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

ICP-MS: Inductively coupled plasma mass spectrometry

IFNγ: Interferon gamma

LC-MS: Liquid chromatography coupled with mass spectrometry

LDL-C: Low-density lipoprotein cholesterol

LDPE: Low-density polyethylene

LNDFHI: Lacto-*N*-difucohexaose I

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

LNT: Lacto-*N*-tetraose

LOD: Limit of detection

LOQ: Limit of quantitation

MCH: Mean corpuscular hemoglobin

MCV: Mean corpuscular volume

ND: Not detected

NHANES: National Health and Nutrition Examination Surveys

NIH: National Institutes of Health

NMR: Nuclear magnetic resonance

NOAEL: No observed adverse effect level

OECD: Organization for Economic Cooperation and Development

PCR: Polymerase chain reaction

Ph Eur: European Pharmacopoeia

pLNnH: para-lacto-*N*-neohexaose

qPCR: Quantitative polymerase chain reaction

RI: Replicative index

TP: Total protein

UDP-Gal: UDP-galactose

UDP-Glc: UDP-glucose

UDP-GlcNAc: UDP-*N*-acetylglucosamine

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

A. SUBMISSION OF GRAS NOTICE

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

B. NAME AND ADDRESS OF THE SPONSOR

Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

C. COMMON OR USUAL NAME

3'-Sialyllactose sodium salt (3'-SL)

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

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

E. INTENDED USE

Jennewein intends to use 3'-SL as a substitute for other forms of 3'-SL in cow's milk non-exempt infant formula for term infants at a level of 0.28 g/L.

F. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of 3'-SL for the intended use and use level specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). The safety of the intake of 3'-SL has been determined to be GRAS by demonstrating that the safety of the intended level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food and is based on generally available and accepted information.

The use of 3'-SL as an ingredient for the intended use in infant formula has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. The subject of this GRAS Notice is a spray-dried, powdered food ingredient that contains not less than 88% 3'-SL dry weight.

    a. 3'-Sialyllactose is a naturally occurring acidic oligosaccharide in human milk.

    b. The 3'-SL that is the subject of this GRAS Notice is structurally identical to the 3'-SL present in human breast milk.

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

    d. The subject of this GRAS Notice is manufactured using a genetically engineered strain of *Escherichia coli* BL21(DE3). Because the host strain does not possess the components required for *E. coli* pathogenicity, strains derived from *E. coli* BL21(DE3) from it are suitable for the production of food ingredients.

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

    f. Fermentation by-products include lactose, sialic acid, and *N*-acetylglucosamine which are known human milk oligosaccharides; their presence in the finished ingredient is not of toxicological concern.

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

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

2. Human milk oligosaccharides, including 3'-SL, are resistant to the digestive enzymes in the gastrointestinal tract, poorly absorbed, and pass through the gastrointestinal tract where they are either fermented by the microbiota or excreted unchanged.

3. Published studies showing that the amount of 3'-SL in breast milk ranges from 0.08 to 0.41 g/L.

4. Genotoxicology and subchronic toxicology studies published by Phipps et al. (2019) show that 3'-SL is not genotoxic and has a NOAEL (no observed adverse effect level) of 5 g/kg bw/day, which was the highest dose tested.

5. The addition of 0.28 g/L 3'-SL in infant formula will result in mean and 90th percentile estimated daily intakes (EDI) of 0.278 g/day (43.1 mg/kg bw/day) and 0.325 g/day (50.4 mg/kg bw/day) for infants 0 to 12 months-of-age.

6. The safety of exposure to Jennewein's 3'-SL at its intended use level is supported by:

    a. Published studies that quantitate the levels of 3'-SL in human milk;

    b. Analytical data demonstrating that the 3'-SL produced by Jennewein is structurally identical to 3'-SL from human milk;

    c. Data demonstrating the qualitative and quantitative similarities between the subject of this GRAS Notice and the 3'-SL ingredient tested by Phipps et al. (2019), which is the subject of GRN 880;

    d. Corroborative published genotoxicology and 90-day subchronic toxicology studies conducted with 3'-SL or a mixture of human milk oligosaccharides containing 4.1 % of Jennewein-manufactured 3'-SL.

7. A corroborative unpublished tolerance study in neonatal piglets conducted with a mixture of HMOs containing up to 0.28 g/L of Jennewein's 3'-SL ingredient that showed an HMO mixture containing 3'-SL was well-tolerated and supported normal growth in neonatal piglets.

Therefore, 3'-SL is safe and GRAS at the proposed level of addition to the intended non-exempt, term infant formula. 3'-Sialyllactose is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

G. PREMARKET APPROVAL

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

H. AVAILABILITY OF INFORMATION

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

I. FREEDOM OF INFORMATION ACT (FOIA)

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

J. INFORMATION INCLUDED IN THE GRAS NOTIFICATION

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

[redacted] 19/3/20

Signature of Authorized Representative of Jennewein Biotechnologie GmbH

Date

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

A. COMMON OR USUAL NAME

3'-Sialyllactose sodium salt (3'-SL; CAS No. 37449-93-7)

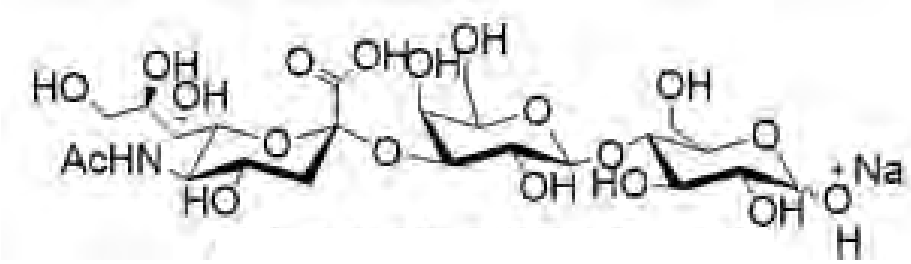
B. CHEMICAL NAME

3'-Sialyl-D-lactose; *N*-acetylneuraminoyllactose sodium salt

C. MOLECULAR FORMULA AND MASS

$C_{23}H_{39}NNaO_{19}$; 633.55Da

D. STRUCTURAL FORMULA



E. DESCRIPTION OF 3'-SIALYLLACTOSE

Approximately 15%-20% of the naturally occurring oligosaccharides (HMOs) found in human milk (the total HMO fraction accounts for 10 to 15 g/L of human milk) are comprised of acidic oligosaccharides. These acidic oligosaccharides contain sialic acid (SA), an acidic sugar with a nine-carbon backbone, and are identified as sialylated HMOs (Bode, 2012). The most recognized sialylated HMOs are the two trisaccharide isomers, 3′- and 6′-sialyllactose, which are both formed as a result of lactose sialylation and account for a significant portion of the acidic HMOs. Both 3′- and 6′-sialyllactose consist of lactose at the reducing terminus and a SA residue at the nonreducing end via an α2,3 or α2,6 bonding, respectively.

The subject of this GRAS Notification, a 3′-sialyllactose sodium salt, is produced by fermentation using a genetically engineered strain of *Escherichia coli* BL21 (DE3). Residual impurities include lactose and carbohydrate by-products. Importantly, the structure of 3'-SL produced by fermentation is consistent with the structure of 3'-SL as confirmed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), ^{1}H, ^{13}C, double-quantum filtered $^{1}H^{1}H$-COSY, phase-sensitive $^{1}H^{13}C$-heteronuclear single quantum correlation (HSQC), phase-sensitive $^{1}H^{13}C$-heteronuclear multiple bond correlation (HMBC) NMR spectroscopy and determined to be equivalent.

Additionally, two 3'-SL sodium salts are GRAS and the subjects of GRAS Notification (GRN) 766 and GRN 880. GRAS Notification 766 received a "no question" letter from FDA on September 26, 2018 and, as of January 21, 2020, FDA's letter for GRN 880 is pending.

F. PRODUCTION PROCESS

The subject of this GRAS Notification is produced by fermentation using *JBT-3SL*, which is a genetically engineered strain of *E. coli* BL21 (DE3). The oligosaccharide is then purified from the fermentation medium and spray dried to form the HMO powder.

1. Description of the Production Strains

To facilitate the engineering of the production strain, a strain known as the Basic strain was engineered as a platform for the subsequent engineering of *JBT-3SL*. All genes integrated into the Basic strain and *JBT-3SL* are well characterized and the resulting strain does not carry plasmids or episomal vectors. The strain is stored at the production site as glycerol stock at -80°C in a master cell bank, which is subsequently used to reproduce working cell banks. Glycerol cultures from the working cell bank are used to directly inoculate fermentation pre-cultures. Additionally, the strain will be deposited at the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen)-German Collection of Microorganisms and Cell Cultures.

a. The Basic Strain

To generate the Basic strain, endogenous genes encoding a β galactosidase, L arabinose-isomerase, L-fucose isomerase, L-fuculokinase, *N*-acetylglucosamine 6-phosphate deacetylase, glucosamine 6 phosphate deaminase, lipopolysaccharide biosynthesis protein, and UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase were either inactivated by mutagenesis using mismatched oligonucleotides or deleted by homologous recombination (Table 1). In contrast, genes encoding a UDP-galactose-4-epimerase, galactosyltransferase, galactokinase, galactose mutarotase, and lactose permease were amplified from *E. coli* K12 genomic DNA and integrated by either site-specific homologous recombination or transposition (Table 1) (Datsenko and Wanner, 2000; Lampe et al., 1999). Arabinose isomerase (*ara*A) 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 integrated during homologous recombination or transposition and used for selection of the recombinants were then removed from the genome by plasmid and Cre-mediated recombination (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 basic strain genomic DNA. Loss of the

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

Table 1. Genetic Manipulations in Basic Strain

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

b. JBT-3SL

In *JBT-3SL*, the endogenous genes encoding *N*-acetylmannosamine kinase, *N*-acetylmannosamine 6-phosphate 2 epimerase, a sialic acid transporter, *N*-acetylmannose lyase, and the phosphophenol pyruvate-dependent mannose specific phosphotransferase system were deleted by homologous recombination, whereas the genes encoding a glutamine fructose-6-phosphate aminotransferase from *E. coli* K12, a glucosamine-6-phosphate *N*-acetyltransferase from *Saccharomyces cerevisiae*, a *N*-acetylglucosamine 2-epimerase from *Synechocystis* sp. PCC6803, a CMP-*N*-acetylneuraminic acid synthase from *Campylobacter jejuni*, a *N*-acetylneuraminic acid synthase from *C. jejuni*, and an α2,3-sialylltransferase derived from *Heamophilus parahaemolyticus* was introduced into the Basic strain by transposition (Table 2). All genomic deletions were performed by site-specific homologous recombination (Datsenko and Wanner, 2000). All ectopically overexpressed genes were synthesized de novo and introduced by transposition (Lampe et al., 1999). The integrants were then subjected nitrosoguanidine (NTG) mutagenesis and screened for their ability to produce high levels of 3'-SL, resulting in *JBT-3SL*. All gene deletions and insertions were verified by PCR using

oligonucleotides specific to the coding sequence and genomic DNA. Loss of all plasmids, which contained antibiotic resistance genes and temperature-sensitive origins of replication, was confirmed by ampicillin sensitivity after incubation at 42°C, and failure to amplify plasmid specific DNA. All integrated genes remain in the genome and, although *JBT-3SL* possesses the dihydrofolate reductase, bleomycin resistance, neomycin-phosphotransferase II, and gentamycin 3'-acetyltransferase genes used for integrant selection, no plasmids or other episomal vectors remain in the genome.

Table 2. Genetic Manipulations in *JBT-3SL*

Gene Product Name	Origin of the Gene	Manipulation	Effect
N-acetylmannosamine kinase	*E. coli* BL21(DE3)	Deletion	To prevent *N*-acetylmannosamine metabolism
N-acetylmannosamine 6-phosphate 2 epimerase	*E. coli* BL21(DE3)	Deletion	
a sialic acid transporter	*E. coli* BL21(DE3)	Deletion	
N-acetylmannose lyase	*E. coli* BL21(DE3)	Deletion	
Phosphotransferase system (PTS) mannose-specific EIIAB component	*E. coli* BL21(DE3)	Deletion	To prevent mannose metabolism
PTS mannose-specific EIIC component	*E. coli* BL21(DE3)	Deletion	
PTS mannose-specific EIID component	*E. coli* BL21(DE3)	Deletion	
Glutamine fructose 6-phosphate aminotransferase	*E. coli* K12	Ectopic expression	To confer *N*-acetyl-D-neuraminic acid and CMP-*N*-acetylneuraminic acid production
Glucosamine 6-phosphate *N*-acetyltransferase	*Saccharomyces cerevisiae*	Ectopic expression	
N-Acetylglucosamine 2-epimerase	*Synechocystis sp.* PCC6803	Ectopic expression	
N-Acetylneuraminic acid synthetase	*Campylobacter jejuni*	Ectopic expression	
CMP *N*-acetylneuraminic acid synthase	*Campylobacter jejuni*	Ectopic expression	
α 2,3-sialylltransferase	*Heamophilus parahaemolyticus*	Ectopic expression	Confer 3'-sailylactose production
Antibiotic resistance genes			
Dihydrofolate reductase conferring resistance to trimethoprim	*Citrobacter freundii*	Ectopic expression	To allow for the selection of integrants during genetic engineering
Neomycin-phosphotransferase II conferring resistance to kanamycin	*Tn5 E. coli* K12	Ectopic expression	
Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	Ectopic expression	
Gentamycin 3'-acetyltransferase conferring resistance to gentamycin	*Acinetobacter baumannii* AYE	Ectopic expression	

2. Manufacturing Process

a. Quality

Production of 3'-SL occurs at the Jennewein Biotechnologie GmbH production facility in Maarweg 32, 53619 Rheinbreitbach, Germany, which is Food Safety System Certification (FSSC) 22000 and ISO 9001:2015 compliant, and a FDA-registered Food Facility (Registration # 1303109037512). Production also occurs at other Jennewein-qualified manufacturers that are GMP-, ISO-, and International Featured Standards Food 6.1-compliant via third party audits.

b. Processing Aids and Food Contact Surfaces

All raw materials, processing aids, and food contact substances used to produce 3'-SL are the same as those used to produce the 2'-FL that is the subject of GRN 571, which received a "no questions" letter from FDA. Therefore, the quality of the processing aids and raw materials and composition of the media described in GRN 571 (pg. 17; Appendix E, pg. 99-144; Appendix J, pg. 280-281) are incorporated by reference. Additional processing aids comply with European Pharmacopoeia, United States Pharmacopeia-National Formulary (USP-NF), or Japanese Pharmacopoeia specifications or appropriate product monographs. The water used throughout the manufacturing process complies with the TrinkwV, 2001 in Germany and the Council Directive 98/83/EC in the European Union and is non-fluoridated drinking water. All food contact surfaces (fermentation vessels and packaging materials) are either stainless steel or comply with the conditions of use that are specified in the US Code of Federal Regulations. The final product is packaged in food grade paper/low-density polyethylene (LDPE) bags in compliance with 21 CFR §177.1520. None of the processing aids are recycled or reused.

c. Production

Except for certain process parameters, 3'-SL is manufactured using the same process as Jennewein's 2'-fucosyllactose that is the subject of GRN 571, which received a "no questions" letter from FDA. The detailed summary of the production process provided in GRN 571 is therefore incorporated by reference (pages 6-9). Briefly, the production of 3'-SL involves three steps (Figure 1). During Step 1, *JBT-3SL* is expanded in minimal medium containing a carbon source consisting of glucose, sucrose, glycerol, or a combination thereof, and then the substrate lactose is added preferentially during the first phase of fermentation resulting in the production and secretion of the oligosaccharide in the culture medium. Step 2 involves purification of the oligosaccharide from the culture medium. Step 3 involves spray- drying of the 3'-SL concentrate into a powder.

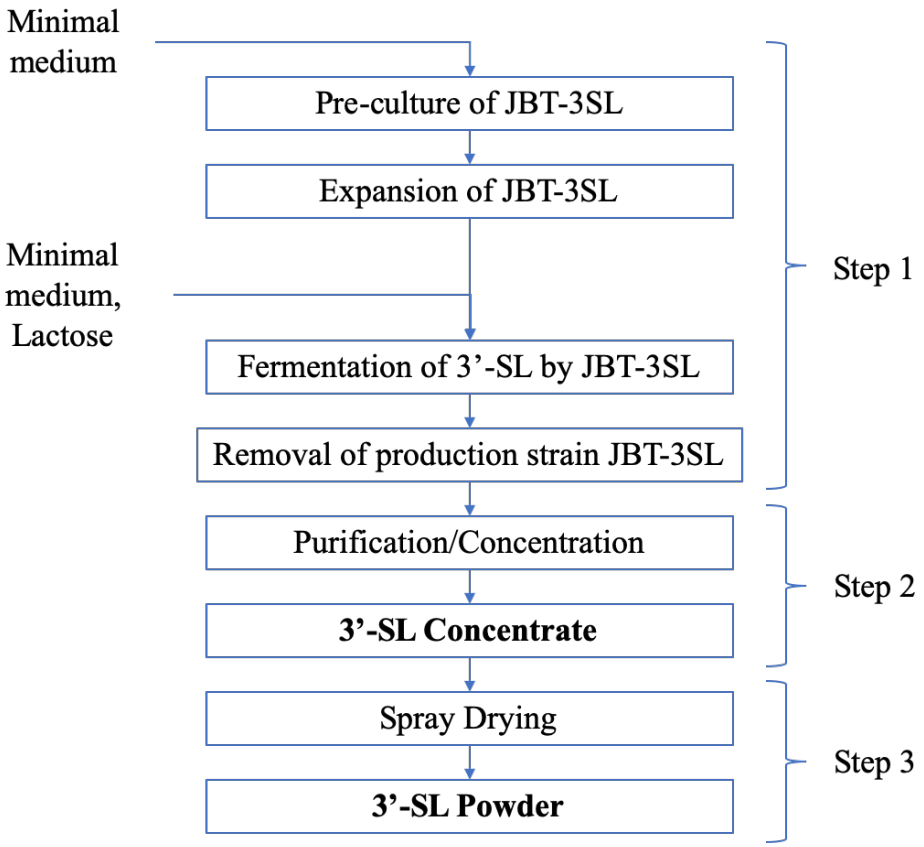


Figure 1. Production Process for 3'-Sialyllactose

JBT-3SL is expanded in minimal medium and with the addition of lactose 3'-SL is produced. The production strain/biomass is removed yielding the 3'-SL -containing fermentation medium. The medium is then purified and concentrated in a series of filtration, ion exchange, electrodialysis, and decolorization steps yielding a 3'-SL concentrate. The concentrate is then spray dried, producing a powder containing 3'-SL.

G. FINISHED PRODUCT SPECIFICATIONS, AND OTHER QUALITY ATTRIBUTES

1. 3'-SL Product Specifications and Batch Data Compliance

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

Data from four non-consecutive batches of 3'-SL show that the manufacturing process reproducibly produces a finished product that complies with the product specifications and removes the production organism from the finished product.

Table 3. Product Specifications and Batch Data 3'-SL

Parameter	Analytical method	Specification	Batch number			
			11027019	11030019	11030029	11031039
Physical Parameters						
Appearance (Color)[4]	Visual	White to ivory-colored	Complies	Complies	Complies	Complies
Appearance (Form)[4]		Spray-dried powder	Complies	Complies	Complies	Complies
Chemical Parameters						
3'-Sialyllactose[4]	HPAEC-PAD	≥ 88% (%DW)	93.9	94.1	93.8	94.5
Other carbohydrates[4]		≤ 12% (% Area)	1.4	1.6	2.0	3.2
Lactose[4]		≤ 5% (% Area)	0.6	0.7	1.0	1.5
Sialic Acid[4]		≤ 10% (% Area)	< LOQ	0.5	0.5	0.7
N-Acetylglucosamine[4]		≤ 5% (% Area)	< LOQ	< LOQ	< LOQ	< LOQ
Protein content[4]	Nanoquant (modified Bradford)	≤ 100 µg/g	14.2	16.3	13.7	18.6
Ash[1]	ASU L 06.00-4	≤ 8.5 %	3.7	4.1	3.4	2.9
Moisture[4]	KF titration	≤ 9.0 %	8.3	8.8	8.5	8.7
Sodium[1]	PV-347 ICP-MS	≤ 4.2 %	3.0	3.1	3.0	3.2
Endotoxins[3]	Ph. Eur. 2.6.14	≤ 10 EU/mg	0.018	0.010	0.011	0.026
Aflatoxin M1[1]	DIN EN ISO 14501	≤ 0.25 µg/kg	< 0.025	< 0.025	< 0.025	< 0.025
GMO residues[2]	PCR	Negative	Negative	Negative	Negative	Negative
Heavy Metals						
Arsenic[1]	ASU L 00.00-135 – ICP-MS	≤ 0.2 mg/kg	ND	ND	ND	ND
Cadmium[1]		≤ 0.1 mg/kg	ND	ND	ND	ND
Lead[1]		≤ 0.02 mg/kg	ND	ND	ND	ND
Mercury[1]		≤ 0.5 mg/kg	ND	ND	ND	ND
Microbiology						
Standard Plate Count[1]	ISO 4833-2	≤ 10000 cfu/g	<10	<10	<10	<10
Yeast and Mold[1]	ISO 21527-2	≤ 100 cfu/g	<20	<20	<20	<20
Enterobacteriaceae[1]	ISO 21528-1	≤ 10 cfu/g	<10	<10	<10	<10
Salmonella [1]	ISO 6579	Absent/25 g	Absent	Absent	Absent	Absent
Cronobacter sakazakii[1]	ISO/TS 22964	Absent/10g	Absent	Absent	Absent	Absent

Abbreviations: DW, dry weight; cfu, colony forming units; STDEV, standard deviation; KF, Karl-Fischer; HPAEC-PAD, high performance anion exchange chromatography coupled with pulsed amperometric detection; qPCR, quantitative polymerase chain reaction; ICP-MS, Inductively coupled plasma mass spectrometry; EU, endotoxin unit; Ph Eur., European Pharmacopoeia; LOQ, limit of quantitation.
[1]Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory; Ash LOQ = 0.01 %. Arsenic limit of detection (LOD) = 0.05 mg/kg; Cadmium LOD = 0.01 mg/kg; Mercury LOD = 0.005 mg/kg; Lead LOD = 0.01 ppm; Aflatoxin M1 LOQ = 0.025 µg/kg.
[2]Determined by GeneCon International GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory. Limit of detection = 0.01% of the finished product.
[3]Determined by Mikrobiologisches Labor. Dr. Michael Lohmeyer GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory; limit of quantitation = 0.005 EU/mg.
[4]Determined by Jennewein Biotechnologie using internally validated methods. Protein LOQ = 10 µg/g; carbohydrate by-products with a percent area greater than 0.5% (limit of quantitation) are considered.

2. Other Quality Attributes

a. Elemental Analyses

Although the oligosaccharide-containing media is subjected to ion exchange chromatography and electrodialysis to minimize the elements in the finished product, Jennewein analyzed three batches of the finished product for the levels of manganese, selenium, iron, copper, molybdenum, nickel, zinc, and cobalt (Table 4). Manganese, iron, molybdenum and cobalt, which are all media components, were all below the limit of detection. Selenium was at or below the limit of quantitation. To ensure that the manufacturing process continues to produce a high-quality finish ingredient, these analyses will be conducted on an annual basis.

Table 4. Elemental Analysis of 3'-Sialyllactose

Element[1]	Method	Batch Number		
		11027019	11030019	11031039
Manganese (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 1.7	< 1.7	< 1.7
Selenium (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.025	< 0.02	< 0.02
Iron (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 0.61	< 0.61	< 0.61
Copper (mg/kg)	ASU L 00.00-135 (ICP-MS)	1.2	1.5	1.5
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)	0.12	0.12	0.12
Zinc (mg/kg)	ASU L 00.00-135 (ICP-MS)	5.5	2.7	2.7
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; selenium LOQ = 0.02 mg/kg; iron LOQ = 0.6 mg/kg; molybdenum LOQ = 0.06 mg/kg; cobalt LOQ = 0.04 mg/kg.

H. STABILITY

1. Genetic stability of the Production Strains

To ensure genomic stability and finished product batch-to-batch consistency, all genes were introduced into the genome of the production strain *JBT-3SL* by either homologous recombination or transposition. Therefore, the strain does not harbor plasmids or episomal vectors. Thus, the production strain is not expected to lose its ability to produce a consistent finished product.

2. Stability of the Finished Product

The shelf-life of 3'-SL is supported by two stability studies conducted on a mixture containing 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-*N*-tetraose (LNT), 3'-SL,

and 6'-sialyllactose (6'-SL) and stability studies conducted on a 3'-SL sodium salt summarized in GRN 766 and 880. Because Jennewein's 3'-SL is compositionally similar to the subject of GRN 766 and 880, it is reasonable to expect the stability of Jennewein's 3'-SL ingredient will be similar. Therefore, the ambient and accelerated stability studies presented in GRN 766 and 880 are incorporated by reference and briefly summarized below, along with the ambient and accelerated stability studies conducted on the 3'-SL-containing mixture of HMOs.

In the ambient stability study (25°C and 25% humidity) conducted on the 3'-SL sodium salt that is subject of GRN 766, purity, appearance, odor, and solubility was conducted on one batch of the finish ingredient over the course of 12 months (GRN 766, pg. 34). No significant change was observed in the 3'-SL sodium salt assay value for up to 12 months of storage. In the ambient stability study (25°C and 60% humidity) conducted on the 3'-SL sodium salt that is subject of GRN 880, chemical, physical, microbiological, and sensory testing has been performed in an ongoing 5-year storage study on 2 representative batches (GRN 880, pg. 21-22). For one batch a complete set of chemical (moisture, 3'-SL, lactose, sialic acid, 3'-sialyl-lactulose, and unspecified impurities), physical (color and appearance), and microbiological (aerobic mesophilic total plate count, *Enterobacteriaceae*, *Salmonella*, *Enterobacter sakazakii*, *Listeria monocytogenes*, *Bacillus cereus*, yeasts, and molds) data was available at 12 months and shows that all parameters tested complied with the product specifications.

In the accelerated stability study (40°C and 25% humidity) conducted on the 3'-SL sodium salt that is subject of GRN 766, purity, appearance, odor, and solubility was conducted on one batch of the finish ingredient over the course of 3 months (GRN 766, pg. 34). No significant change was observed in the 3'-SL sodium salt assay value for up to 3 months under accelerated storage conditions. In the accelerated stability study (40°C and 75% humidity) conducted on the 3'-SL sodium salt that is subject of GRN 880, chemical, physical, microbiological, and sensory testing has been performed in an ongoing 2-year storage study on 2 representative batches (GRN 880, pg. 23). For one batch a complete set of chemical (moisture, 3'-SL, lactose, sialic acid, 3'-sialyl-lactulose, and unspecified impurities), physical (color and appearance), and microbiological (aerobic mesophilic total plate count, Enterobacteriaceae, Salmonella, *Cronobacter sakazakii*, *Listeria monocytogenes*, *Bacillus cereus*, yeasts, and molds) data was available at 12 months and shows that all parameters tested complied with the product specifications.

Jennewein's HMO mixture contained approximately 5% 3'-SL by dry weight after production and was stored in the high-density polyethylene bottles under ambient (25°C and 60% relative humidity) and accelerated (40°C and 75% relative humidity) conditions for 52 and 26 weeks, respectively. 3'-SL and moisture content were monitored over time using the same methods

that are used for batch qualification. Although there was analytical variability 3'-SL content remained relatively unchanged over the course of the 52-week testing period under ambient conditions. Moisture content increased from 5.7 to 7.8% (Table 5). Under accelerated conditions, 3'-SL decreased, and moisture increased over the course of the 26-week testing period (Table 6).

Thus, together these results support a shelf-life for 3'-SL of 1 year from the date of production when stored under ambient conditions.

Table 5. Stability of 3'-Sialyllactose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Ambient Conditions (25°C, 60% Relative Humidity)

Batch 4011-1004303107		Moisture		3'-SL	
		%	% of baseline	% DW	% of baseline
Interval	Baseline	5.7	100.0	4.98	100
	Week 1	5.2	91.9	5.01	100.4
	Week 4	6.2	109.2	4.74	95.1
	Week 8	6.1	108.3	5.20	104.3
	Week 13	6.1	107.2	5.25	105.2
	Week 26	6.9	121.7	5.16	103.4
	Week 39	7.3	129.3	4.84	97.0
	Week 52	7.8	137.0	4.92	98.7

Abbreviations: DW, dry weight; 3'-SL, 3'-sialylactose; NA, not applicable.

Table 6. Stability of 3'-Sialyllactose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Accelerated Conditions (40°C, 75% Relative Humidity)

Batch 4011-1004303107		Moisture		3'-SL	
		%	% of baseline	% DW	% of baseline
Interval	Baseline	5.7	100.0	4.98	100
	Week 1	5.8	101.4	5.32	106.7
	Week 4	6.6	117.1	4.70	94.2
	Week 8	7.3	129.1	5.16	103.4
	Week 13	8.7	153.6	5.28	105.8
	Week 26	9.9	174.6	4.96	99.4

Abbreviations: DW, dry weight; 3'-sialyllactose; NA, not applicable.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

The intended effect of adding 3'-SL powder to term, non-exempt infant formula is to increase 3'-SL intake in formula-fed infants and promote the growth of beneficial bacteria, including, but not limited to, *Bifidobacteria*.

B. HISTORY OF EXPOSURE

3'-Siallylactose is a naturally occurring acidic oligosaccharide found in human and bovine milk. Synthetic forms of 3'-SL have also been approved for use in infant formula (GRN 766, 880). Thus, humans are predominantly exposed to 3'-SL either through the ingestion of breast milk, cow's milk-based infant formulas, and/or products containing synthetic forms of 3'-SL.

Acidic oligosaccharides make up 15-20% of all HMOs found in human milk (Bode, 2012a). Generated from lactose or other non-sialylated or non-fucosyllated-HMOs by sialyltransferases, sialyllactose predominantly exists as either 3'-SL or 6'-SL. The concentration of 3'-SL in human breast milk has been analyzed by about 22 major studies (Aakko et al., 2017; Asakuma et al., 2007; Austin et al., 2016; Azad et al., 2018; Bao et al., 2007; Coppa et al., 1999; Gabrielli et al., 2011; Goehring et al., 2014; Hong et al., 2014; Kunz et al., 2017; Leo et al., 2010; Ma et al., 2018; Martin-Sosa et al., 2003; McGuire et al, 2017; Nakhla et al., 1999; Nijman et al, 2018; Olivares et al., 2015; Smilowitz et al., 2013; Sprenger et al., 2017; Spevacek et al., 2015; Sumiyoshi et al., 2003; Thurl et al., 2010). Many of these studies were included in the recent systematic review conducted by Thurl et al. (2017). A summary of the results presented by Thurl et al. and additional studies not reviewed in Thurl et al. (2017) are presented in Table 7. In the available studies, the average concentration of 3'-SL ranged from 0.08-0.41 g/L. Unlike other HMOs, such as 2'-FL and 3-FL, 3'-SL concentrations do not differ between Secretor status of the mother. Longitudinal studies have demonstrated that 3'-SL concentration stays relatively constant with lactation time (Austin et al., 2016; Kunz et al., 2017; Ma et al., 2018; Sprenger et al., 2017) and although there is some minor variability, 3'-SL levels are relatively consistent across different geographical regions (McGuire et al., 2017).

3'SL is also found in bovine milk at concentrations ranging from 0.092-0.867 g/L (Lee et al., 2015; McJarrow et al., 2004; Nakamura et al., 2003). Unlike in human milk, 3'-SL levels in bovine milk decrease over lactation time and has been found to vary amongst bovine species.

Synthetic forms of 3'-SL have also been approved for use in infant formula at levels up to 0.238 g/L, 1.6 g/kg for use in baby food products, 25 g/kg in foods for special dietary use, and up to 12.5 g/kg in conventional foods and beverages (GRN 766, 2018; GRN 880).

Table 7. Studies Determining the Concentration of 3'-Siallylactose in Human Breast Milk				
Study	**Location**	**Number of Subjects/Samples**	**Timepoint(s)**	**3'-SL concentration**
Aakko et al., 2017	Finland	11 donors	• 24 hours	• Mean: 0.413 ± 0.09 g/L • Range: not provided
Austin et al., 2016	China	446 donors	• Days 5-11 • Days 12-30 • Months 1-2 • Months 2-4 • Months 4-8	• Range: 0.043-0.26 g/L • Highest mean: 0.11 ± 0.035 g/L (Days 5-11; median 0.11 g/L) • Lowest mean: 0.079 ± 0.020 g/L (Months 4-8; median 0.077 g/L)
Azad et al., 2018	Canada	427 donors	• 3-4 months post-partum (mature milk)	• Mean: 0.36 g/L ± 0.23 g/L • Median: 0.32 g/L • Range: 0.060-2.89 g/L
Kunz et al., 2017	Spain	32 donors, 96 samples	• Days 1-7 (colostrum) • Days 8-15 (transitional milk) • Days 16-30 (mature milk)	• Highest median: 0.28 g/L (Days 1-7) • Lowest median: 0.18 g/L (Days 16-30) • Range: 0.16-0.34 g/L
Leo et al., 2010	Samoa	16 donors	• Days 5-10 • Days 22-155	• Highest mean: 0.16 ± 0.11 g/L (Days 5-10) • Lowest mean: 0.13 ± 0.056 g/L (Days 22-155) • Range: 0.023-0.317 g/L
Ma et al., 2018	China, Malaysia	China: 20 donors Malaysia: 26 donors	China • Days 14, 30, 60, 90, 120, 180, and 240 post-partum Malaysia: • Days 2, 60, 180, and 365 post-partum	• Highest mean: 0.22 ± 0.083 (Day 2 Malaysian mothers) • Lowest mean: 0.099 ± 0.021 (Day 60 Chinese mothers) • Range: 0.038-0.43 g/L

Table 7. Studies Determining the Concentration of 3'-Siallylactose in Human Breast Milk				
Study	**Location**	**Number of Subjects/Samples**	**Timepoint(s)**	**3'-SL concentration**
McGuire et al, 2017	Around the World	410 donors	• Weeks 2-5	• Highest mean: 0.39 ± 0.035 g/L (Ghana) • Lowest mean: 0.26 ± 0.021 g/L (rural Ethiopia) • Range: not provided
Nijman et al., 2018	United States	10 donors, 20 samples	• Day 3 • Day 42	• Highest mean: 0.12 ± 0.00 g/L (Day 42) • Lowest mean: 0.11 ± 0.01 g/L (Day 3) • Range: not provided
Sprenger et al., 2017	Indonesia	50 donors	• Day 30 • Day 60 • Day 120	• Highest mean: 0.26 ± 0.088 g/L (Day 30 low 2'-FL group; median 0.24 g/L) • Lowest mean: 0.20 ± 0.060 g/L (Day 60 high 2'FL group; median 0.19 g/L) • Range: 0.026-0.44 g/L
Thurl et al., 2017	Around the World	200 donors; 509 samples	• Days 0-100	• Mean concentration: 0.16 g/L (0.12-0.19 g/L 95% CI)
2'-FL: 2'-fucosyllactose; 3'-SL: 3'-siallylactose; CI: confidence interval				

C. INTENDED USES

Jennewein intends to use 3'-SL sodium salt as a substitute for other forms of 3'-SL in cow's milk based, non-exempt infant formula for term infants at a level of 0.28 g/L. Although this level is approximately 1.17-fold greater than what has been determined GRAS (GRN 776 - 0.238 g/L infant formula), the results reported in the available studies that quantitated the levels of 3'-SL in breast milk indicate that the increased level of use will adequately accommodate variations in 3'-SL levels.

D. ESTIMATED DAILY INTAKE

Because Jennewein intends to use its 3'-SL sodium salt as a substitute for other forms of 3'-SL currently marketed in the United States at a level approximately 1.17-fold greater than what is GRAS, the resulting mean and 90th percentile estimated daily intakes of 3'-SL from infant formula that were provided in GRN 766 (pg. 44) will increase from 0.187 g/day (25.9 mg/kg bw/day) and 0.278 g/day (43.1 mg/kg bw/day) to 0.219 g/day (30.3 mg/kg bw/day) and 0.325 g/day (50.4 mg/kg bw/day) in infants 0 to12 months of age.

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 3'-SL under the specified conditions of use in non-exempt term infant formula is based on the following: the published studies that have quantitated the levels of 3'-SL in human milk (see Section III.B); the analytical data demonstrating that the 3'-SL produced by Jennewein is structurally identical to 3'-SL from human milk; the published toxicology studies with Glycom's 3'-SL ingredient, which support the GRAS status of the subject of GRN 880; the qualitative and quantitative similarities between the subject of this GRAS Notice and Glycom A/S's 3'-SL ingredient; the corroborating toxicology studies conducted with HMO mixtures containing Jennewein-manufactured 3'-SL; and other corroborating neonatal piglet and clinical studies that evaluated the tolerability of 3'-SL.

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 all of the essential nutrients for healthy growth and development and is believed to promote protection from infection (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 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., 2012b). 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).

3'-Sialyllactose is a naturally occurring oligosaccharide in human milk. Numerous published studies have examined the level of 3'-SL in human milk, and the reported range is 0.08-0.41 g/L. Unlike other HMOs, such as 2'-FL and 3-FL, 3'-SL concentrations do not differ between Secretor status of the mother and stays relatively constant with lactation time. Jennewein's use of 3'-SL at 0.27 g/L is well within the established range of 3'-SL that naturally occurs in breast milk.

Because human milk is the reference standard for infant nutrition, infant formula manufacturers look to mimic the composition and functionality of human milk in their formulas

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

Additionally, the use of selected HMOs opposed to a mixture of the almost 200 HMOs in infant formula has been called into question (Milani et al., 2017). However, it is important to note that breast milk is considered to be the reference standard for infant nutrition, both the types and amounts of HMOs in breast milk can vary greatly from one mother to another, and observational studies that investigated the effects of varying breast milk HMO composition on infant growth and health have reported conflicting results due to design limitations and/or confounding factors (Alderete et al., 2015; Azad et al., 2018; Berger et al., 2020; Lagström et al., 2020; Gridneva et al., 2019; Kuntz et al., 2019; Larsson et al., 2019; Sprenger et al., 2017; Vandenplas et al., 2018). Thus, a clear and consistent link between the use of selected and structurally different HMOs in infant formula and adverse outcomes on infant growth and health does not exist. Therefore, based on the totality of the available evidence, it is reasonable to expect that supplementing infant formula with a synthetic form of 3'-SL will not pose risks to infants consuming formula containing 3'-SL.

3'-SL is the subject of two GRAS notifications 766, which received a "no questions" letter from the FDA, and 880, which is still pending. Pivotal genotoxicity studies that support the safety of 3'-SL as a single ingredient in both notifications include two bacterial reverse mutation assays, an *in vitro* chromosomal aberration test in CHO cells, an *in vitro* mammalian micronucleus test, and an *in vivo* micronucleus test (Kim et al., 2018; Phipps et al., 2019). Jennewein also conducted a bacterial reverse assay and an *in vitro* micronucleus test on an HMO mixture, including 4.1% 3'-SL by dry weight (Parschat et al., 2020). In all cases, 3'-SL was determined to be non-genotoxic.

The safe use of Jennewein's 3'-SL ingredient in infant formula is supported by a battery of published and unpublished genotoxicity, subchronic toxicity, and tolerability studies (Phipps et al., 2019; Kim et al., 2018; Parschat et al., 2020; unpublished neonatal piglet study). Because Jennewein's 3'-SL ingredient is manufactured in a similar manner, and qualitatively similar and quantitatively comparable to the 3'-SL ingredient manufactured by Glycom A/S and tested by

Phipps et al., (2019), the genotoxicity and subchronic toxicity studies conducted by Phipps et al. are the pivotal studies that support the safe use of Jennewein's 3'-SL product. 3'-Sialyllactose is not genotoxic and has a no observed adverse effect level (NOAEL) of at least 5.0 g/kg bw/day, which was the highest dose tested in a 90-day subchronic toxicity study. Additional genotoxicity, and acute and subchronic toxicity studies conducted by Kim et al. (2018) and Parschat et al. (2020), which were conducted with a 3'-SL product manufactured by GeneChem and a mixture of HMOs containing 3'-SL manufactured by Jennewein, respectively, corroborate the results reported by Phipps et al. 2019, as well as an unpublished neonatal piglet study conducted with a mixture of HMOs containing 3'-SL manufactured by Jennewein.

Thus, based on the totality of available safety data, there is reasonable certainty that Jennewein's 3'-SL is of no harm to consumers per the intended use and use level. Jennewein's 3'-SL is therefore GRAS as an ingredient in term, non-exempt infant formula at the intended use level.

A. SAFETY OF THE PRODUCTION ORGANISM

The safety of the host organism, *E. coli* BL21(D3), is thoroughly summarized in GRN 485 (pg. 15-18), GRN 571 (Appendix K, pg. 282-300), and the GRN 571 Supplement, all of which received "no questions" letters from the FDA. GRN 485 and 571 describe the use of *E. coli* BL21(D3) as the host organism in the production of BbgIV *beta*-galactosidase and 2'-FL, respectively.

Escherichia coli are commensal residents of the gut microflora of humans and numerous animal species. *E. coli* strains are taxonomically grouped into 5 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 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, the use of *E. coli* BL21(DE3) is not expected to result in any safety concerns.

B. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

The absorption, distribution, metabolism and excretion (ADME) of HMOs has been extensively summarized in previous GRAS Notices and opinions published by authoritative bodies around the world (GRN 484, 2014; GRN 546, 2015; GRN 547, 2014; GRN 571, 2015; GRN 650, 2016; GRN 659, 2016; GRN 735, 2018; GRN 749, 2018; GRN 766, 2018; GRN 815, 2019; GRN 833, 2019; EFSA Panel on Dietetic Products, 2015; EFSA Panel on Nutrition et al., 2019). Briefly, HMOs, including LNT, are highly resistant to the digestive enzymes of the gastrointestinal (GI) tract and only small amounts are absorbed intact. *In vitro* studies have shown that <5% of ingested HMOs is digested. *In vivo* studies among infants and in rats have reported that 1 to 2% of the total amount of ingested HMO is excreted unchanged in urine and the remaining unabsorbed oligosaccharides then pass through the gastrointestinal tract where it is either fermented by the select resident microbiota or excreted unchanged in the feces (Goehring et al., 2014; Ruhaak et al., 2014; Santos-Fandila et al., 2014; Dotz et al., 2014; Obermeier et al., 1999; Rudloff et al., 2012; Rudloff et al., 2006; Rudloff and Kunz, 2012; Rudloff et al., 1996; Chaturvedi et al., 2001; Gnoth et al., 2000; Engfer et al., 2000; Brand-Miller et al., 1998). Although the exact mechanisms by which HMO absorption occurs have not been fully elucidated, data from *in vitro* studies using the Caco-2 human intestinal epithelial cell line suggest that neutral HMOs, such as LNT, are transported across the intestinal epithelium by receptor-mediated transcytosis as well as by paracellular transport, whereas acidic HMO, such as 3'-SL, are absorbed via the non-specific paracellular transport only (Gnoth et al., 2000).

C. TOXICOLOGY

The pivotal genotoxicity and subchronic toxicity of 3'-SL that support the use of Jennewein's 3'-SL ingredient in infant formula include a battery of genotoxicity and subchronic toxicity studies conducted using a 3'-SL-containing ingredient manufactured by Glycom A/S and published by Phipps et al. (2019). Additional corroborating genotoxicity and subchronic toxicity studies published by Kim et al. (2018) and Parschat et al. (2020) were conducted using a 3'-SL ingredient manufactured by GeneChem Inc using enzymatic synthesis and a mixture containing

2'-FL, 3'-FL, LNT, 3'-SL, 6'-SL, all of which are manufactured by Jennewein using carefully controlled fermentation conditions, respectively.

Specifically, Phipps et al. (2019) conducted an OECD-compliant bacterial reverse mutation assay, an OECD-compliant *in vitro* micronucleus assay, and an OECD-compliant 90-day subchronic oral toxicity study in neonatal rats with a product containing 90.3% 3'-SL manufactured by Glycom A/S to support the GRAS status of the subject of GRN 880. Kim et al (2018) conducted an OECD-compliant bacterial reverse mutation assay, an OECD-compliant *in vitro* chromosome aberration assay, an *in vivo* micronucleus assay, an acute toxicity study in rats, a 28 day subchronic toxicity study in rats, an OECD-complaint 90-day subchronic oral toxicity studies in rats, a single dose escalation study in Beagle dogs with a product containing 98.8% 3'-SL manufactured by GeneChem, Inc. to support the GRAS status of the subject of GRN 766. Parschat et al. (2020) evaluated the genotoxicity and subchronic toxicity of Jennewein's 3'-SL in combination with 2'-FL, 3'-FL, LNT, and 6'-SL, in an OECD-compliant bacterial reverse mutation assay, an OECD-compliant *in vitro* micronucleus assay, a seven-day pilot dietary toxicity study and an OECD-compliant 90-day feeding study. Importantly, both the 3'-SL sodium salts tested by Phipps et al. (2019) and Parschat et al. (2020) are manufactured by fermentation using genetically engineered strains of *E. coli*, contain similar amounts of 3'-SL, and have comparable carbohydrate by-products and other impurities, which are controlled by product specifications (Table 8). As stated in GRN 880, lactose and sialic acid are the major degradation products of 3'-SL. Additionally, 3'-sialyl-lactulose, which is a possible degradation product of Glycom A/S's 3'-SL ingredient that is the subject of GRN 880 and was used by Phipps et al. (2019), is not expected in Jennewein's product due to the 3'-SL production process.

Table 8. Comparison of Jennewein's 3'-Sialyllactose Sodium Salt With the 3'-Sialyllactose Sodium Salts Tested by Phipps et al., 2019 and That Supports GRN 880

Parameter	GRN 880		Jennewein's Specifications
	Phipps et al., 2019[a]	Specifications	
3'-sialyllactose	90.3 %	≥ 88.0 %	≥ 88.0%
Sodium	3.07 %	2.5 - 4.5 %	≤ 4.2%
Lactose	0.63 %	≤ 5 %	≤ 5%
Sialic acid	0.48 %	≤ 1.5 %	≤ 10%
3'-Sialyl-lactulose	1.70 %	≤ 5 %	NS
N-acetylglucosamine	NR	NS	≤ 5%
Others	1.08 %	≤ 3 %	NS
Sum of other carbohydrates	3.89 %[b]	NS	≤ 12%[c]
Protein	NR	≤ 100 µg/g	≤ 100 µg/g
Ash	NR	NS	≤ 8.5%
Moisture	3.2%	≤ 8 %	≤ 9.0%

3-FL: 3-fucosyllactose; LOQ: limit of quantitation; NR: not reported; NS: not specified.
[a]Supports the GRAS status of the subject of GRN 880.
[b]Includes lactose, sialic acid, 3'sialyllactulose and others.
[c]Includes lactose, sialic acid, and *N*-acetylglucosamine.

Therefore, because Jennewein's 3'-SL product is qualitatively comparable and quantitatively similar to the 3'-SL ingredient tested by Phipps et al. (2019), the results of those genotoxicology and subchronic toxicology studies are pivotal to supporting the safety of Jennewein's 3'-SL product. Additionally, although they tested a 3'-SL ingredient manufactured by enzymatic synthesis and a mixture containing lower amounts of 3'-SL, the results reported by Kim et al. (2018) and Parschat et al. (2020) corroborate the findings reported by Phipps et al. (2019). Briefly, 3'-SL is not genotoxic, has a NOAEL of 5000 mg/kg bw/day, the highest dose tested in subchronic toxicity studies, and when administered as an HMO mixture at 10% of the diet results in a NOAEL of 5.67 g/kg bw/day (0.23 g/kg bw/day of 3'-SL) for male rats and 6.97 g/kg bw/day (0.29 g/kg bw/day of 3'-SL) for the female rats.

1. Genotoxicity Studies

a. Genotoxicity Studies of 3'-SL as a Single Ingredient

Genotoxicity studies are summarized in the latest GRN 880 (pg. 41-42 and pg. 45-46) and are incorporated by reference. In summary, two OECD-compliant bacterial reverse mutation assays and an OECD-compliant chromosomal mutation assay conducted with 3'-SL ingredients manufactured by Glycom A/S and GeneChem, Inc. showed 3'-SL to be non-mutagenic and non-clastogenic at concentrations up to 5000 µg/mL (Kim et al., 2018; Phipps et al., 2019). Additionally, the genotoxicity of the Glycom A/S 3'-SL ingredient was also tested in an OECD-compliant *in vitro* micronucleus assay and the 3'-SL ingredient manufactured by GeneChem, Inc. was tested in both an OECD-compliant *in vitro* micronucleus assay and an OECD-compliant *in vivo* micronucleus assay. In all three micronucleus assays, the 3'-SL ingredients were shown to be not clastogenic or aneugenic up to 2000 mg/kg bw (Kim et al., 2018; Phipps et al., 2019).

b. Genotoxicity Studies of Jennewein's 3'-SL as Part of an HMO Mixture

i. Bacterial Reverse Mutation Test

To evaluate the mutagenicity of an HMO mixture containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein using fermentation, Parschat et al. (2020) conducted an OECD 471-complaint bacterial reverse mutation test. Five strains of *S. typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) were used in two independent experiments with and without metabolic activation. The first experiment was conducted as a plate incorporation test and the second as a preincubation test (Ames et al., 1973; Ames et al, 1975; Maron and Ames, 1983). Five, 10.0, 31.6, 100, 316 or 600 mg of the mixture containing 0.21, 0.41, 1.3, 4.1, 13.0, and 24.6 mg 3'-SL, respectively, were applied to each plate. Purified water was the negative control and the positive controls for the different strains were

sodium azide (for TA1535 and TA100), 2-nitrofluorene (for TA98), benozo[a]pyrene 9AA (for TA1537, and mitomycin C (for TA102). Cytotoxicity was defined as a reproducible reduction in the number of colonies by more than 50% compared to the solvent control and/or a scarce background lawn.

Compared to the negative control, the positive controls increased the mean revertant colony numbers at least threefold with and without metabolic activation (Table 9), verifying the validity of the test. For the HMO mixture, no cytotoxicity or mutagenicity were noted in any of test strains up to 600 mg HMO mixture/plate (equivalent to 24.6 mg 3'-SL/plate) in either the plate incorporation or preincubation tests (Table 9). Parschat et al. concluded that the results indicate that the HMO mixture, and the 3'-SL contained therein, was not mutagenic under the conditions tested.

Table 9. Bacterial Reverse Mutation Test Performed With an HMO Mixture Containing 4.1% 3'-Sialyllactose[c]

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

Abbreviations: BaP, benozo[a]pyrene; 2-AA, 2-aminoanthracene; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; NaN_3, sodium azide.
Values are means (n=3) ± standards deviations.
[a]Positive controls without S9: NaN_3 for TA1535 and TA100, 2-NF for TA98, 9-AA for TA1537, mitomycin C for TA102.
[b]Positive controls with S9: BaP for TA98, TA102 and TA1537, 2-AA for TA100 and TA1535.
[c]The HMO mixture containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein.

ii. Micronucleus Test

To evaluate the clastrogenicity and/or aneugenicity of an HMO mixture containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein using fermentation, Parschat et al. (2020) performed an OECD 487-compliant *in vitro* micronucleus test using human peripheral blood lymphocytes. Peripheral blood lymphocytes were obtained by venipuncture from young, healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation and exposed to 7.5, 15, 30, and 60 mg HMO mixture/mL medium (equivalent to 0.31, 0.62, 1.23, and 2.46 mg 3'-SL/mL medium) for 4 or 24 hrs in the presence and absence of metabolic activation. Purified water was the negative control and the positive controls were mitomycin C (at 0.2 μg/mL), colchicine (at 0.02 μg/mL), and cyclophosphamide (at 20 μg/mL) with and/or without metabolic activation. At least 500 cells per replicate cell culture were scored and classified as mononucleates, binucleates, or multinucleates to estimate the proliferation index as a measure of toxicity. The evaluation of cytotoxicity was based on the Cytokinesis-Block Proliferation Index (CBPI) or the Replicative Index (RI). The CBPI indicates the average number of nuclei per cell during the period of exposure to CytoB and is used to calculate cell proliferation. The RI indicates the relative number of cell cycles in treated cultures compared to control cultures and can be used to calculate the percentage of cytostasis. Micronucleus frequencies were analyzed in at least 2000 binucleate cells per concentration (i.e., ≥ 1000 binucleate cells per culture; two cultures per concentration). The ability of the HMO mix to induce micronuclei was considered to be positive if there was a statistically significant and/or dose related increase compared to the negative control or if any of the results were outside the distribution of the historical negative control data (Poisson-based 95% control limits).

Mitomycin C and cyclophosphamide induced significant chromosomal damage whereas colchicine induced significant ($p \leq 0.05$) damage to the cell division apparatus, both validating the tests. In contrast, no chromosomal damage was observed with the HMO mixture at any concentration or under any condition tested (Table 10). Thus, the HMO mixture was not genotoxic under the tested conditions at concentrations up to 60 mg/mL (2.46 mg/mL 3'-SL).

Table 10. *In vitro* Micronucleus Test in Human Peripheral Blood Lymphocytes Exposed to an HMO Mixture Containing 4.1% 3'-Sialyllactose[b]

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

CBPI = Cytokinesis block proliferation index; RI = Replicative Index.
Values are means (n = 2).
[a]Significantly different from negative control ($p \leq 0.05$).
[b]The HMO mixture also contained 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein.

2. Toxicology Studies of 3'-SL as a Single Ingredient

a. Toxicity Studies

The animal toxicity studies that support the safe use of 3'-SL are summarized in GRN 880 (pg. 37-41, pg. 42-44), are incorporated by reference. In summary, an acute single dose toxicity study in rats showed that the lethal dose (LD_{50}) for the 3'-SL manufactured by GeneChem, Inc. was above 20 g/kg bw, the highest dose tested; a 28-day subchronic oral toxicity study in rats showed that there were no treatment-related abnormalities body weight gain, feed consumption, clinical chemistry, hematology, organ weights, relative organ weights, or histopathology for the 3'-SL ingredient manufactured by GeneChem, Inc. at doses up to 2 g/kg/day; a subchronic 90-day oral toxicity studies in rats conducted with the 3'-SL ingredient manufactured by Glycom A/S established a NOAEL of up to 5 g/kg bw, the highest dose tested; a subchronic 90-day oral toxicity studies in rats conducted with the 3'-SL ingredient manufactured by GeneChem, Inc. also established a NOAEL of up to 5 g/kg bw, the highest dose

tested; and an acute single escalating-dose oral toxicity study in Beagle dogs conducted with the 3'-SL manufactured by GeneChem, Inc. showed that the Maximum Tolerated Dose (MTD) was greater than 2 g/kg bw/day (Kim et al., 2018; Phipps et al., 2019).

3. Toxicity Studies on Jennewein's 3'-SL as Part of an HMO Mixture

a. Seven-day Dietary Toxicity Study

In a seven-day pilot dietary toxicity study, female CD/Crl:CD rats (Charles River Laboratories, Sulzfeld, Germany) received either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Jennewein for 7 days (n=5/group) (Parschat et al., 2020). The HMO mixture contained 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL and 5.1% dry weight other carbohydrates, all of which were manufactured by fermentation. Thus, the overall dietary exposure to 3'-SL was 0.4 % of the diet. Both diets were provided *ad libitum.* Animals were observed daily for viability, behavioral changes, and reactions to treatment or illness. Cage-side observations included skin and fur, eyes, mucous membranes, respiratory and circulatory systems, somatomotor activity, behavior patterns, and feces output and consistency. Body weight was recorded at the time of group allocation, on the 1st day of treatment and daily thereafter at the same time each day. Feed consumption was recorded daily and feed intake per rat (g/rat/day) was calculated subtracting the total amount of feed left from the total amount of feed given and dividing the difference by the number of days and body weight of the rat. Drinking water consumption was monitored daily by visual inspection. Intake of the test article was calculated on a daily and weekly basis throughout the experimental period based on the concentration in the diet, individual food intake and body weight of each rat. No mortalities occurred during the study. No HMO-related differences in behavior, appearance and consistency of the feces, body weight, body weight gain, or feed consumption were observed. Thus, the dose of 10% HMO mixture in diet (4.1% 3'-SL by dry weight, providing 3'-SL as 0.41% of total diet) was chosen for the subsequent 13-week oral toxicity study in rats.

b. Subchronic Dietary Toxicity Study

Parschat et al. (2020) fed either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Jennewein to rats for 90 days (n=10/sex/group) in an OECD 408-compliant 90-day dietary toxicity study. The HMO mixture contained 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL and 5.1% dry weight other carbohydrates, all of which were manufactured by fermentation. Thus, the overall dietary

exposure to 3'-SL was 0.41% of diet. Both diets were provided *ad libitum.* All animals were individually housed, and observed daily for clinical signs of toxicity and twice daily for mortality. Cage-side observations included changes in the skin, fur, eyes and mucous membranes, the occurrence of secretions or excretions, autonomic activity (e.g. lacrimation, pilo-erection, pupil size, and unusual respiratory patterns), gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling) or bizarre behaviors (e.g. self- mutilation, walking backwards). Clinical observations were made once before the first exposure and weekly thereafter. Body weight was recorded at the start of the adaptation period, at the time of group allocation, on the day treatment commenced, and weekly thereafter at the same time each day. Feed consumption was recorded daily, and feed intake per rat (g/rat/week) and relative feed consumption (g/kg bw/day) were calculated. Drinking water consumption was monitored daily by visual inspection. Neurological screening was conducted in test week 13 before blood sampling to evaluate sensory reactivity to different stimuli (auditory, visual and proprioceptive stimuli), grip strength and to assess locomotor activity. Observational screening included tests covering peripheral, sensory, muscular, central and gastro-intestinal neural components. Functional tests comprised grip strength and locomotor activity. Ophthalmological and auditory examinations were conducted before the study and one week before the end of treatment. Blood and urine samples were taken from overnight fasted animals at the end of test week 13 before necropsy. Blood was collected for hematology, coagulation, and clinical chemistry analyses. Urine was collected for 16 hours and analyzed for volume, pH, specific gravity, protein, glucose, bilirubin, urobilinogen, ketones, hemoglobin, and nitrite. Urine was also analyzed by microscopy for epithelial cells, leucocytes, erythrocytes, organisms, crystalluria, and constituents such as sperm and casts. Color and turbidity of the urine were examined visually.

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

Based on feed consumption, the mean intake of the HMO mixture ranged from 5.01 to 6.88 g/kg bw/day for male rats and 6.26 to 7.91 g/kg bw/day for the female rats. This resulted in a mean intake of 3'-SL of 0.21 to 0.28 g/kg bw/day in males and 0.26 to 0.32 g/kg bw/day in females.

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

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

range for the laboratory (224 and 299 movements/12 min vs an upper boundary of 217 movements/12 min; laboratory historical control mean of 77.7 movements/12 min). Thus, the increase in body temperature and decrease in spontaneous mobility were deemed to be incidental and not related to the HMO mixture.

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

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

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

Table 11. Statistically Significant Differences in Clinical Chemistry Values on Day 92

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

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

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

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

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

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

Table 12. Significant Differences in Mean Brain and Kidney Weights

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

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

Table 13. Significant Differences in Mean Relative Kidney Weights

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

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

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

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

Overall, no signs of toxicity were observed following the administration of an HMO mixture (containing 4.1% 3'-SL by dry weight) at 10% of diet for 13 weeks. Based on feed intake data, the NOAEL for this study was 5.67 g/kg bw/day for male rats and 6.97 g/kg bw/day for the female rats. This resulted in a mean intake of 3'-SL of 0.23 g/kg bw/day in males and 0.29 g/kg bw/day in females.

D. TOLERANCE STUDY IN NEONATAL PIGLETS

1. Introduction

To understand the safety and tolerance of a mixture containing Jennewein-manufactured HMOs (2'-FL, 3-FL, LNT, 3'-SL and 6'-SL; oligosaccharide blend) in a species other than the rat, a 21 day-neonatal piglet study was conducted. One animal died during the study due to a non-HMO-related bacterial infection. The mixture was well tolerated and did not produce any adverse effects on growth, development, hematology, clinical chemistry, organ weights, gross pathology or histopathology at levels up to 8 g/L. Although this study has not been published, the results corroborate the lack of adverse effects seen in the subchronic chronic oral toxicity study conducted by Phipps et al. (2019) with 3'-SL and the subchronic rat dietary toxicity study conducted by Parschat et al. (2020) using a mixture of HMOs that contained 3'-SL.

2. Materials and Methods

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

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

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

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

Control and Test Articles: The control used in the study was ProNurse® (Land O'Lakes Purina Feed, LLC) mixed with deionized water. The test article was an "oligosaccharide blend" containing 49.1 % 2'-FL, 10.4 % 3-FL, 19.9% LNT, 3.5 % 3'-SL and 4.17 % 6'-SL, resulting in a total oligosaccharide content of 87%. Formulations of the test article were prepared by mixing the appropriate amount of ProNurse® with the appropriate amount of test article to achieve nominal concentrations of 5.75 and 8 g/L, which resulted in 2.8 g 2'-FL/L, 0.6 g 3-FL/L, 1.2 g LNT/L, 0.2 g 3'-SL/L, and 0.2 g 6'-SL/L in the 5.75 g/L formulation and 3.9 g 2'-FL /L, 0.8 g 3-FL/L, 1.6 g LNT/L, 0.3 g 3'-SL/L, and 0.3 g 6'-SL/L in the 8 g/L formulation. Both formulations were prepared daily and stored refrigerated at 2°C to 8°C. Dosing formulations prepared for the study were evaluated for homogeneity and concentration by collecting samples from the top, bottom, and middle of the formulations using a syringe and 150 mm (Day 1) or 24 inch (Day 20) sampling tube, while stirring, and quantifying the total amount of HMOs.

Administration of Test Materials: Starting on the day of receipt (Day 1), the control and test articles were offered orally via a feeding container 6 times per day (3 hours ±15 minutes

between each dose) at a dose volume of 500 ml/kg/day for up to 21 days. The control group received the control article in the same manner as the treated groups.

The study design was as follows (Table 14):

Table 14. Experimental Design

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

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

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

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

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

Clinical Pathology: Hematology, coagulation, clinical chemistry and urinalysis sample collection was performed as detailed in Table 15.

Table 15. Clinical Pathology Sample Collection Plan

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

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

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

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

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

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

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

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

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

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

3. Results

Dose Formulation Analyses: Homogeneity and concentration analyses results of the 5.75 and 8.0 g/L formulations prepared on Day 1 and Day 20 ranged from 93.3% to 94.1%, respectively, of the targeted dose levels and confirmed that formulations were homogenous and animals received the appropriate concentrations (Table 16).

Table 16. Analysis of Total Oligosaccharide Content in Dosing Formulations – Days 1 and 20

Dose Level (g/L)	Average Calculated Concentration (g/L)[a]	Average % Recovery[a]
0	0.07-0.15	NA
5.75	5.37 – 5.41	93.4 – 94.1
8.0	7.46 – 7.51	93.3 – 93.9

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

Clinical Observations: No test article-related clinical findings were observed at any of the Oligosaccharide Blend dose levels evaluated. The few clinical findings observed in the treated groups were either similar to those observed in concurrent controls and/or seen infrequently and/or considered common in animals of this species, strain, and age and unrelated to treatment (Table 17). Discolored yellow/watery feces were noted in piglets from all groups and a systemic antibiotic (LA200 (oxytetracycline injectable solution)) was administered for a period of 3 days during the study to piglets exhibiting a fecal score of 6 (no form, watery texture, and watery composition). A total of 5/12 (4 males and 1 female), 4/12 (2 male and 2 female) and 5/11 (3 male and 2 female) piglets were treated in the control, 5.7 g/L and 8.0 g/L groups, respectively (Table 18).

Table 17. Summary of Detailed Clinical Observations						
Observation Type: All Types	**Males**			**Females**		
From Day 3 (Start Date) to 21 (Start Date)	**0 g/L**	**5.75 g/L**	**8.0 g/L**	**0 g/L**	**5.75 g/L**	**8.0 g/L**
Total Number of Animals	6	6	6	6	6	6
EXCRETION						
Feces discolored, Yellow						
Number of Times Recorded	3	5	2	0	2	1
Number of Animals Affected	2	2	2	-	2	1
Feces soft						
Number of Times Recorded	0	2	1	0	0	0
Number of Animals Affected	-	2	1	-	-	-
Feces watery						
Number of Times Recorded	0	4	1	0	1	1
Number of Animals Affected	-	2	1	-	1	1
EXTERNAL APPEARANCE						
Discharge, Red						
Number of Times Recorded	0	0	0	1	3	1
Number of Animals Affected	-	-	-	1	1	1
Material around eyes, Black						
Number of Times Recorded	4	2	0	2	0	0
Number of Animals Affected	2	1	-	1	-	-
Swelling						
Number of Times Recorded	0	1	2	1	1	0
Number of Animals Affected	-	1	1	1	1	-
Thin						
Number of Times Recorded	1	1	2	0	0	0
Number of Animals Affected	1	1	1	-	-	-
EYE/OCULAR						
Eyelid part/completely closed						
Number of Times Recorded	0	0	3	0	0	0
Number of Animals Affected	-	-	2	-	-	-
PELAGE/SKIN						

Table 17. Summary of Detailed Clinical Observations						
Observation Type: All Types	**Males**			**Females**		
From Day 3 (Start Date) to 21 (Start Date)	**0 g/L**	**5.75 g/L**	**8.0 g/L**	**0 g/L**	**5.75 g/L**	**8.0 g/L**
Abrasion(s)						
Number of Times Recorded	4	1	0	2	14	4
Number of Animals Affected	2	1	-	1	4	1
Scabbed area						
Number of Times Recorded	13	13	3	11	37	5
Number of Animals Affected	4	3	2	4	4	3
Skin discolored, Red						
Number of Times Recorded	2	2	6	3	6	3
Number of Animals Affected	2	2	2	2	2	2
EXCRETION						
Emesis, White						
Number of Times Recorded	2	0	0	0	0	0
Number of Animals Affected	2	-	-	-	-	-
Emesis, Yellow						
Number of Times Recorded	1	0	0	0	0	0
Number of Animals Affected	1	-	-	-	-	-
Feces discolored, Orange						
Number of Times Recorded	0	0	1	0	0	0
Number of Animals Affected	-	-	1	-	-	-
Vomitus, Yellow						
Number of Times Recorded	0	0	0	1	0	0
Number of Animals Affected	-	-	-	1	-	-
PELAGE/SKIN						
Skin warm to touch						
Number of Times Recorded	0	0	0	0	1	0
Number of Animals Affected	-	-	-	-	1	-
Unkempt appearance						
Number of Times Recorded	1	0	1	0	0	0
Number of Animals Affected	1	–	1	-	-	-

Table 18. Piglets Receiving Antibiotic (LA200 (oxytetracycline injectable solution)) During the Study

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

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

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

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

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

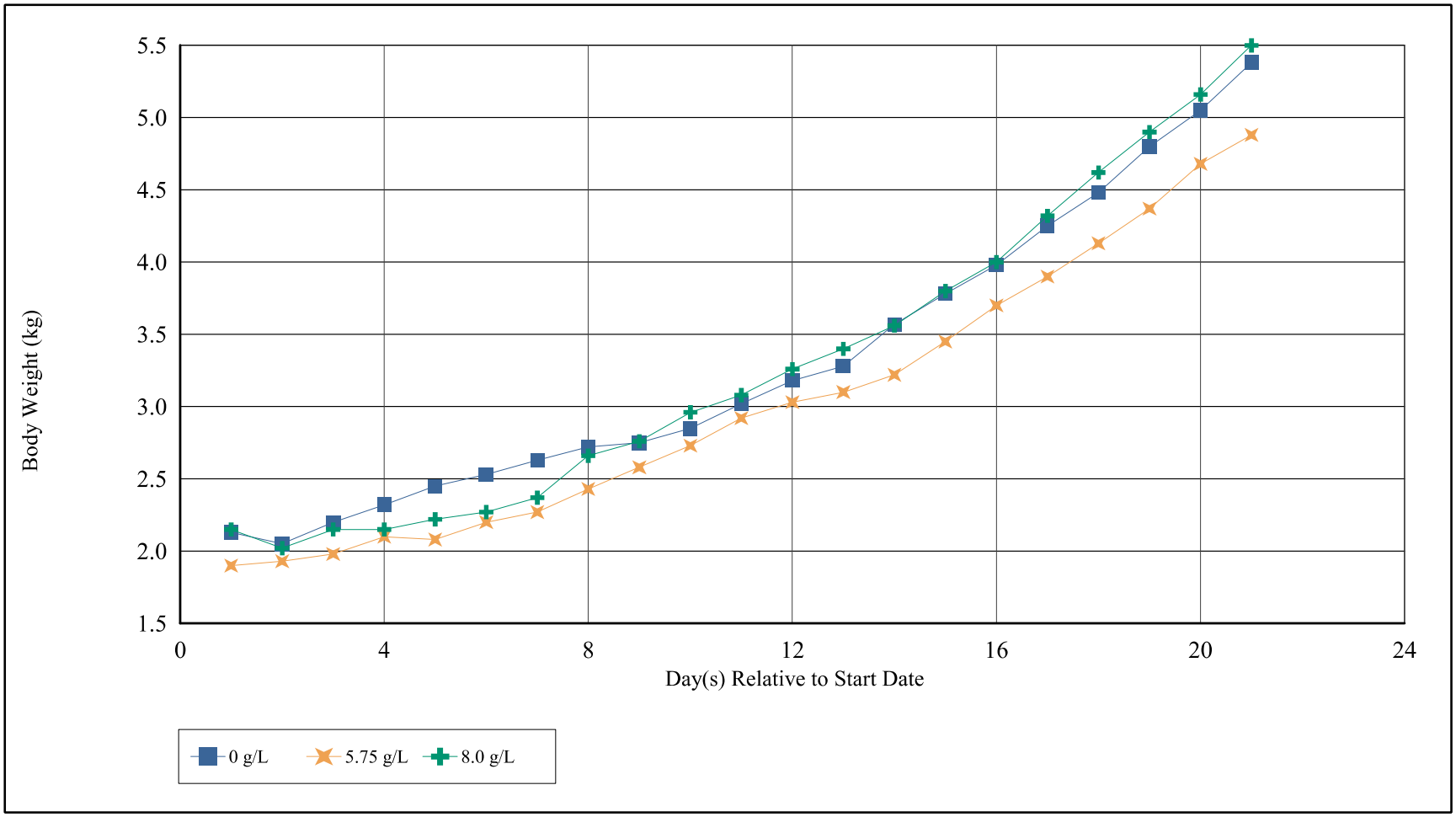


Figure 2a. Mean Body Weight Values (Male)

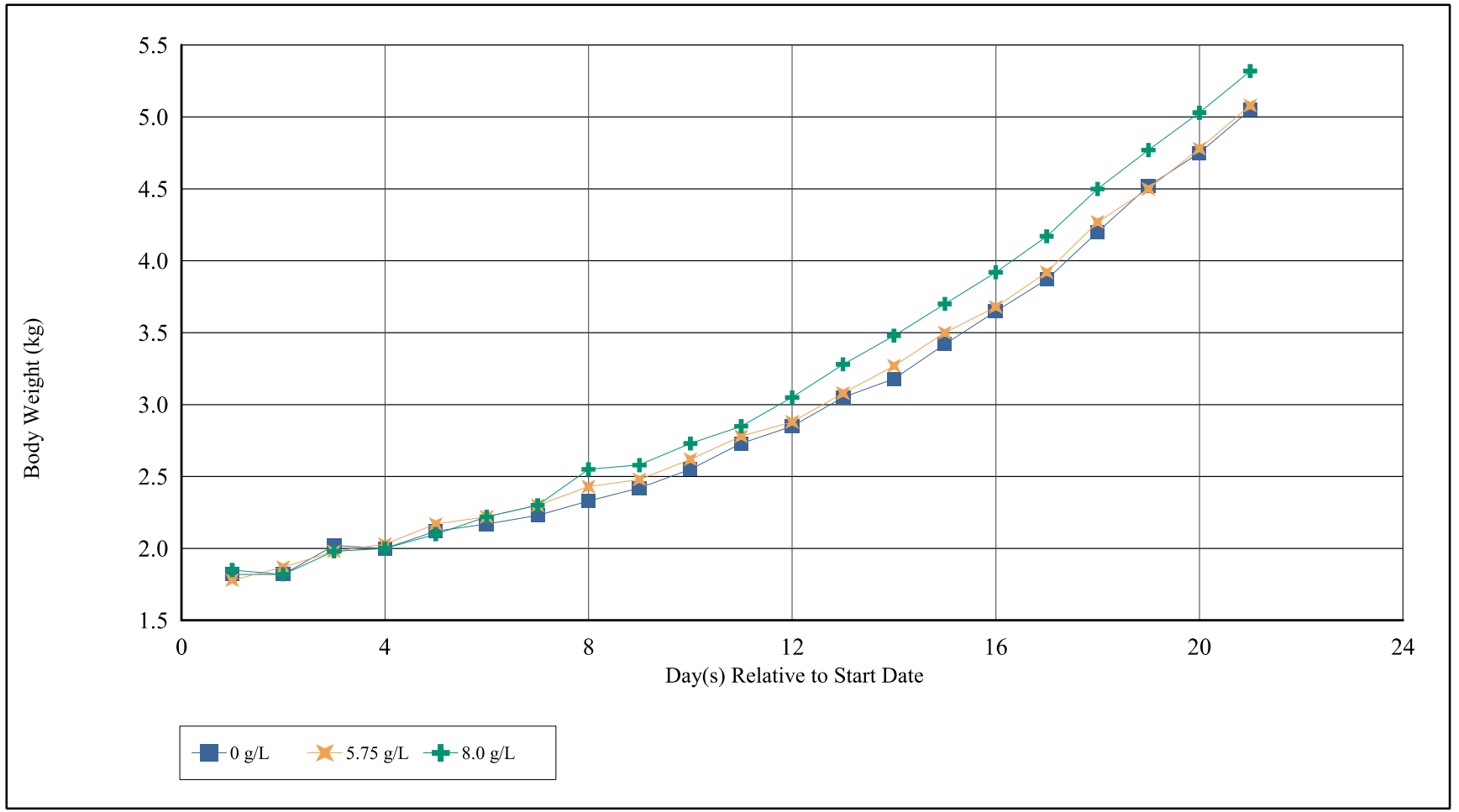


Figure 2b. Mean Body Weight Values (Female)

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

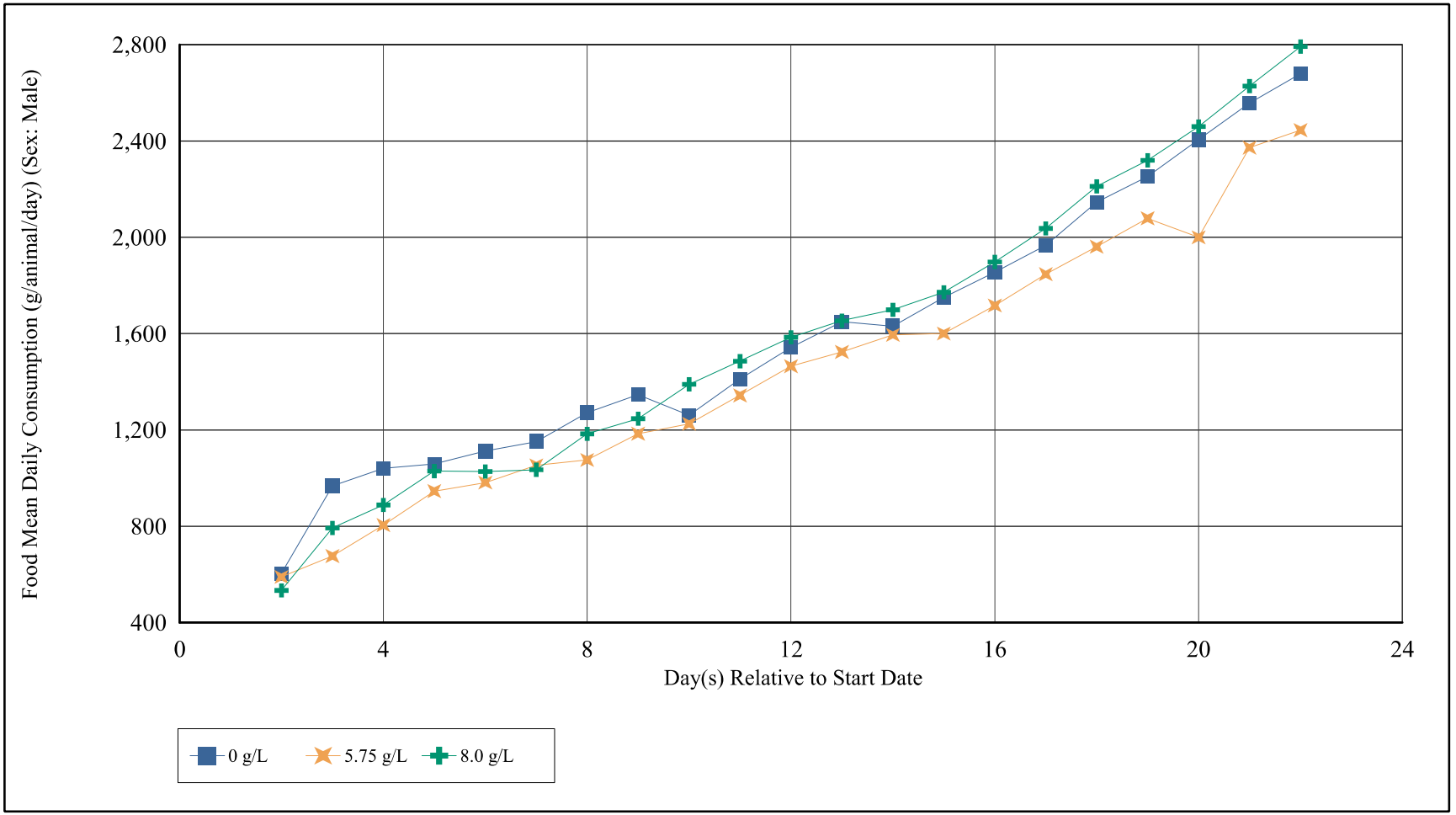


Figure 3a. Mean Feed Consumption Values (Male)

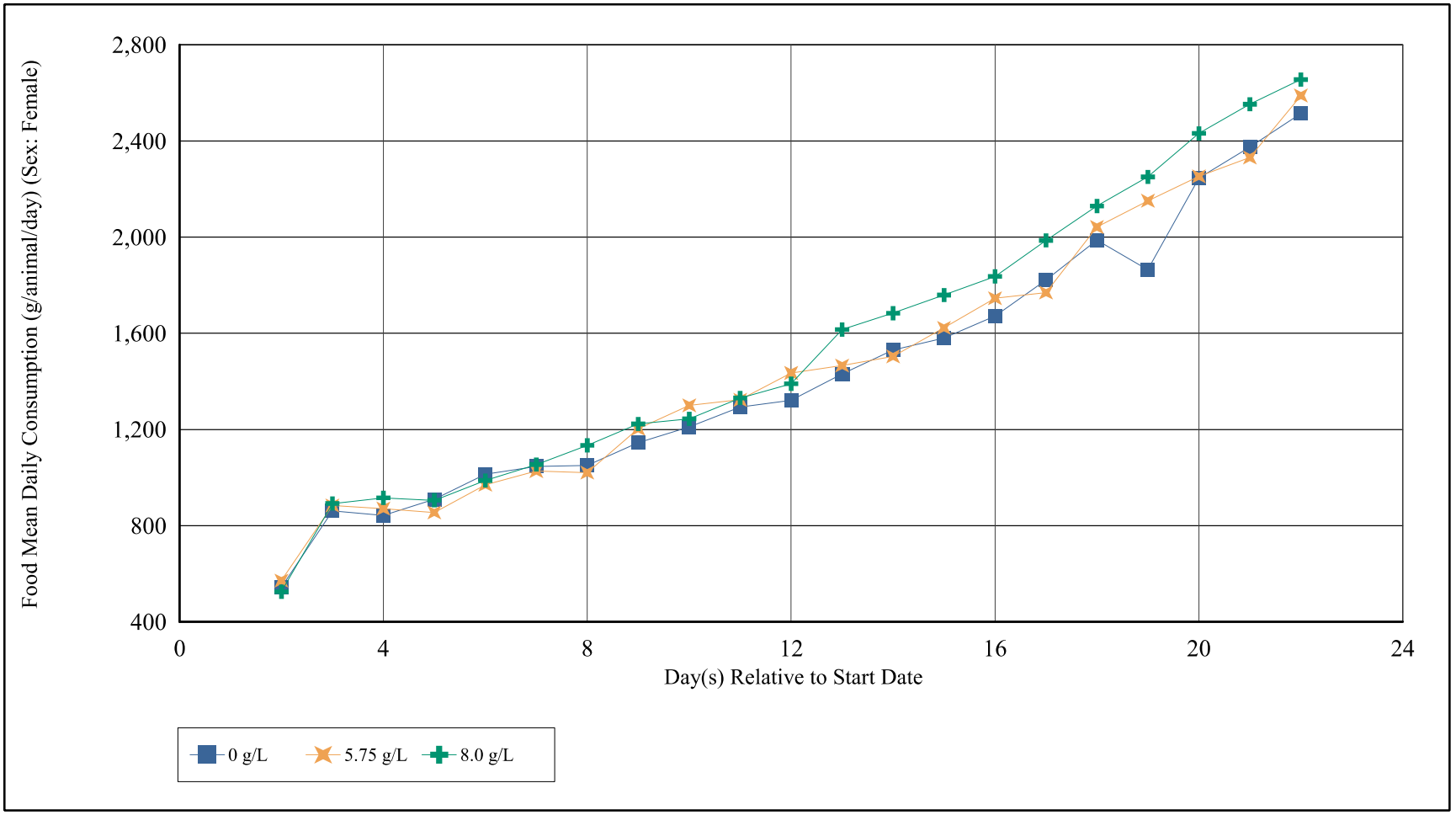


Figure 3b. Mean Feed Consumption Values (Female)

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

Table 21. Feed Efficiency (Mean % ± St. Dev (n))

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

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

Clinical Pathology:

Hematology: Administration of Oligosaccharide Blend in the diet did not result in test article-related hematological changes (Table 22). Although hematological changes were observed in one male at 8.0 g/L (Animal No. 3001) that was euthanized on Day 7, the changes were incidental and not treatment-related. Other differences in the hematological parameters, were not considered related to Oligosaccharide Blend administration based on their small magnitude, inconsistent direction, absence of a dose response, general overlap of individual values with the range of control values, and/or were of a magnitude of change commonly observed in farm piglets under similar study conditions.

Coagulation: Administration of Oligosaccharide Blend in the diet did not result in test article-related coagulation changes in APTT, prothrombin time or fibrinogen in males or females. All differences in coagulation parameters, regardless of statistical significance, were not considered related to oligosaccharide blend administration based on their small magnitude, inconsistent direction, absence of a dose response, general overlap of individual values with the range of control values, and/or were of a magnitude of change commonly observed in farm piglets under similar study conditions (Table 23).

Clinical chemistry: Administration of Oligosaccharide Blend in the diet did not result in test article-related clinical chemistry changes (Table 24).

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

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

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

Urinalysis: Administration of Oligosaccharide Blend in the diet did not result in test article-related urinalysis changes (Table 25).

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

Table 22. Hematology (Mean ± St Dev (n))

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

Table 22. Hematology (Mean ± St Dev (n))

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

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

[g] – ANOVA & Dunnett (Log)

[g1] – ANOVA & Dunnett

[g2] – Kruskal-Wallis & Dunn

Table 23. Coagulation Parameters (Mean ± St Dev (n))

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

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

Table 24. Clinical Chemistry (Mean ± St Dev (n))							
Parameter	**Day**	**Male**			**Female**		
		0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
Sodium (mEq/L)	7 [g]	138.7 ± 3.59 (6)	138.9 ± 2.71 (6)	140.4 ± 5.70 (6)	139.6 ± 3.23 (6)	138.6 ± 2.12 (6)	140.8 ± 1.11 (6)
	21 [g]	143.8 ± 1.33 (6)	144.2 ± 3.03 (6)	142.9 ± 1.21 (5)	141.8 ± 2.00 (6)	143.6 ± 1.59 (6)	144.8 ± 1.94 (6)[a]
Potassium (mEq/L)	7 [g1]	6.64 ± 0.531 (6)	6.52 ± 0.723 (6)	6.56 ± 1.884 (6)	6.60 ± 0.607 (6)	6.63 ± 0.632 (6)	6.51 ± 0.704 (6)
	21 [g]	6.77 ± 0.506 (6)	6.70 ± 0.424 (6)	6.44 ± 0.421 (5)	6.20 ± 0.734 (6)	6.32 ± 0.459 (6)	6.67 ± 0.527 (6)
Chloride (mEq/L)	7 [g2]	101.5 ± 3.11 (6)	102.8 ± 3.75 (6)	107.7 ± 12.31 (6)	103.3 ± 2.42 (6)	102.1 ± 2.54 (6)	103.0 ± 1.76 (6)
	21 [g2]	105.8 ± 1.41 (6)	105.4 ± 1.99 (6)	104.4 ± 0.57 (5)	104.7 ± 1.29 (6)	105.2 ± 1.97 (6)	105.7 ± 1.10 (6)
Calcium (mg/dL)	7 [g2]	10.86 ± 0.303 (6)	10.92 ± 0.511 (6)	10.85 ± 1.063 (6)	10.85 ± 0.619 (6)	11.07 ± 0.575 (6)	11.28 ± 0.223 (6)
	21 [g]	10.87 ± 0.234 (6)	11.03 ± 0.296 (6)	10.85 ± 0.093 (5)	10.52 ± 0.268 (6)	10.84 ± 0.235 (6)	10.92 ± 0.197 (6)a
Phosphorus (mg/dL)	7 [g1]	8.32 ± 0.676 (6)	8.08 ± 0.598 (6)	8.46 ± 1.938 (6)	8.74 ± 1.017 (6)	8.39 ± 0.913 (6)	8.51 ± 0.551 (6)
	21 [g]	10.31 ± 0.861 (6)	10.19 ± 1.224 (6)	10.19 ± 0.563 (5)	10.21 ± 1.096 (6)	10.26 ± 0.606 (6)	10.61 ± 0.794 (6)
ALP (U/L)	7 [g1]	444.0 ± 182.21 (6)	886.5 ± 704.82 (6)	509.4 ± 266.21 (6)	491.3 ± 193.73 (6)	618.9 ± 162.27 (6)	457.7 ± 156.73 (6)
	21 [g2]	486.6 ± 64.21 (6)	498.6 ± 142.62 (6)	471.8 ± 115.17 (5)	623.3 ± 259.77 (6)	618.2 ± 175.40 (6)	412.4 ± 54.82 (6)
Total Bilirubin (mg/dL)	7 [g1]	0.22 ± 0.067 (6)	0.29 ± 0.187 (6)	0.18 ± 0.040 (6)	0.23 ± 0.097 (6)	0.19 ± 0.087 (6)	0.18 ± 0.070 (6)
	21 [g]	0.14 ± 0.026 (6)	0.15 ± 0.021 (6)	0.15 ± 0.030 (5)	0.15 ± 0.016 (6)	0.15 ± 0.008 (6)	0.15 ± 0.034 (6)
GGT (U/L)	7 [g2]	27.9 ± 14.68 (6)	31.6 ± 12.08 (6)	35.8 ± 3.32 (6)	24.5 ± 8.52 (6)	25.3 ± 6.49 (6)	29.6 ± 6.32 (6)
	21 [g]	21.0 ± 8.72 (6)	24.4 ± 8.30 (6)	26.7 ± 5.37 (5)	18.8 ± 6.24 (6)	20.9 ± 4.09 (6)	30.9 ± 19.64 (6)
AST (U/L)	7 [g1]	62.0 ± 62.10 (6)	32.8 ± 7.34 (6)	31.3 ± 15.78 (6)	32.6 ± 2.92 (6)	34.4 ± 13.64 (6)	36.8 ± 11.21 (6)
	21 [g]	31.8 ± 5.46 (6)	33.9 ± 5.78 (6)	36.5 ± 7.41 (5)	42.0 ± 18.80 (6)	32.9 ± 6.65 (6)	50.8 ± 22.62 (6)
ALT (U/L)	7 [g1]	28.0 ± 10.52 (6)	20.9 ± 2.76 (6)	23.1 ± 3.31 (6)	28.7 ± 4.02 (6)	24.2 ± 4.03 (6)	23.3 ± 7.05 (6)
	21 [g]	23.3 ± 5.21 (6)	22.7 ± 4.23 (6)	25.1 ± 2.29 (5)	24.5 ± 5.90 (6)	22.7 ± 5.04 (6)	24.2 ± 4.56 (6)
SDH (U/L)	7 [g]	3.77 ± 3.288 (3)	4.68 ± 1.024 (4)	1.47 ± 0.603 (3)	0.70 ± - (1)[n]	1.18 ± 0.512 (4)[n]	2.68 ± 1.546 (4)[n]
	21 [I]	1.20 ± 0.707 (2)[n]	1.28 ± 0.631 (6)[n]	2.07 ± 1.159 (3)[n]	1.10 ± 0.141 (2)[n]	2.18 ± 1.668 (4)[n]	1.33 ± 0.737 (3)[n]
Urea Nitrogen (mg/dL)	7 [g1]	9.3 ± 2.22 (6)	9.6 ± 5.59 (6)	28.0 ± 49.70 (6)	9.9 ± 3.34 (6)	5.9 ± 2.57 (6)	5.7 ± 3.02 (6)
	21 [g]	6.4 ± 0.86 (6)	6.3 ± 1.26 (6)	5.0 ± 1.03 (5)	6.9 ± 0.94 (6)	5.2 ± 1.24 (6)[a]	5.3 ± 1.02 (6)[a]
Creatinine (mg/dL)	7 [g2]	0.51 ± 0.132 (6)	0.53 ± 0.035 (6)	0.87 ± 0.892 (6)	0.46 ± 0.077 (6)	0.52 ± 0.109 (6)	0.53 ± 0.046 (6)

Table 24. Clinical Chemistry (Mean ± St Dev (n))

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

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

[g] – ANOVA & Dunnett

[g1] – Kruskal-Wallis & Dunn

[I] – n = Inappropriate for statistics

a = $p < 0.01$

Table 25. Urinalysis (Mean ± St. Dev (n))

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

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

Organ Weights: Absolute and/or relative cecum weights increased dose-dependently in males and females at ≥5.75 g/L with statistical significance limited to relative cecum/body weight percentage in males at 8.0 g/L (Table 26). No microscopic correlates were observed to account for the increased cecum weights.

Table 26. Summary of Large Intestinal Weight Data – Scheduled/Terminal Euthanasia (Day 22)

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

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

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

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

A summary of the other absolute and relative organ weights is shown in Table 27. Other differences in organ weight parameters were attributed to normal biologic variation. These differences had no patterns, trends, or correlating data to suggest these differences were test article related.

Table 27. Absolute and Relative Organ Weights

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

Table 27. Absolute and Relative Organ Weights

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

Table 27. Absolute and Relative Organ Weights							
Organ	**Parameter**	**Male**			**Female**		
		0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
Large intes, [g] rectum/BWt (%)	Mean ± SD (n)	0.24747 ± 0.104625 (6)	0.24031 ± 0.133841 (6)	0.17353 ± 0.022982 (5)	0.24757 ± 0.093317 (6)	0.23808 ± 0.125050 (6)	0.17172 ± 0.018482 (6)
	%Diff	-	-2.9	-29.9	-	-3.8	-30.6
Large intes, [g] rectum/BrWt (ratio)	Mean ± SD (n)	0.30346 ± 0.165604 (6)	0.27319 ± 0.165705 (6)	0.21312 ± 0.056898 (5)	0.27318 ± 0.080039 (6)	0.27524 ± 0.150411 (6)	0.20743 ± 0.038094 (6)
	%Diff	-	-10.0	-29.8	-	0.8	-24.1
Liver w/ [g] Gallbladder (g)	Mean ± SD (n)	181.5603 ± 22.06378 (6)	170.0287 ± 29.61167 (6)	189.6808 ± 36.37935 (5)	186.0467 ± 30.35304 (6)	182.7653 ± 28.28351 (6)	189.5793 ± 22.68564 (6)
	%Diff	-	-6.4	4.5	-	-1.8	1.9
Liver w/ GB [g] /BWt (%)	Mean	3.30938 ± 0.317106 (6)	3.33397 ± 0.231707 (6)	3.42467 ± 0.244156 (5)	3.58558 ± 0.407498 (6)	3.40645 ± 0.336352 (6)	3.49212 ± 0.519496 (6)
	%Diff	-	0.7	3.5	-	-5.0	-2.6
Liver w/ GB [g] /BrWt (ratio)	Mean	3.91412 ± 0.468559 (6)	3.76490 ± 0.679597 (6)	4.16844 ± 0.765231 (5)	4.06531 ± 0.576787 (6)	3.94377 ± 0.611197 (6)	4.16009 ± 0.508501 (6)
	%Diff	-	-3.8	6.5	-	-3.0	2.3
Small intes. [g] Duodenum (g)	Mean ± SD (n)	62.3568 ± 13.72859 (6)	56.8028 ± 15.81976 (6)	61.9216 ± 11.33367 (5)	61.2420 ± 15.35857 (6)	63.3058 ± 13.22122 (6)	62.9915 ± 16.85156 (6)
	%Diff	-	-8.9	-0.7	-	3.4	2.9
Small intest [g] duodenum/BWt (%)	Mean ± SD (n)	1.12189 ± 0.108449 (6)	1.09742 ± 0.181502 (6)	1.12029 ± 0.119668 (5)	1.16656 ± 0.184777 (6)	1.17408 ± 0.159768 (6)	1.14063 ± 0.159768 (6)
	%Diff	-	-2.2	-0.1	-	0.6	-2.2
Small intest [g] duoden/BrWt (ratio)	Mean ± SD (n)	1.34402 ± 0.289943 (6)	1.25607 ± 0.356362 (6)	1.36372 ± 0.258269 (5)	1.33719 ± 0.315534 (6)	1.36546 ± 0.283729 (6)	1.38965 ± 0.401617 (6)
	%Diff	-	-6.5	1.5	-	2.1	3.9

Table 27. Absolute and Relative Organ Weights

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

Table 27. Absolute and Relative Organ Weights

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

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

[g] – ANOVA & Dunnett

[g1] – ANOVA & Dunnett (Log)

[g2] – Kruskal-Wallis & Dunn

A = $p < 0.05$

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

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

4. Discussion

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

F. CORROBORATIVE ANIMAL STUDIES

Additional neonatal piglet studies have further corroborated the safety of the consumption of 3'-SL (Obelitz-Ryom et al., 2018; unpublished piglet study summarized in GRN 766 (pg. 53-54); Monaco et al., 2018; Monaco et al., 2019; Wang et al., 2019; Obelitz-Ryom et al., 2019). Although these studies focused on the effect of sialyllactose on brain and gut development, as well as effects on the microbiome, none reported adverse effects related to sialyllactose and 3'-SL supplementation. Only the endpoints relevant to the safety and tolerability of sialyllactose and 3'-SL supplementation are briefly summarized below.

Obelitz-Ryom et al. (2018) fed preterm piglets intact unpasteurized Jersey cow's milk supplemented with either GOS or 4.5% sialyllactose (a 6:1 ratio of 3'-SL and 6'-SL) for 19 days and assessed gut development and colonization. No adverse events related to the experimental diet were reported in the study, and there were no differences in body weight gain between the treatment groups. There were no differences in serum biochemistry or phagocytic capacity of neutrophils observed between the two treatment groups.

An unpublished piglet study described in GRN 766 (pg. 53-54), concluded that 3'-SL sodium salt at concentrations of 140, 200, or 500 mg/L was well tolerated and supported normal growth and development.

Monaco et al. (2018) fed 2-day old male piglets increasing doses of sialyllactose (130, 380, or 760 mg sialyllactose/L milk replacer; 3' or 6' isomer was not specified) for 30 days to investigate the effect of sialyllactose on weight gain, gastrointestinal development and microbiota composition. No differences were observed among the treatment groups in body weight gain over the test period. Although some differences were observed among treatment groups in hematology parameters, these differences were within the historical background range for this species and laboratory and were not considered treatment-related or adverse. There were no changes observed in clinical chemistry parameters among the treatment groups with the exception of glutamate dehydrogenase. This difference was not dose dependent and was not considered treatment related or adverse.

Monaco et al. (2019) fed piglets a control diet or a diet supplemented with 140, 200, or 500 mg/L 3'-SL for 21 days. The concentrations correspond to 135.3, 193.3 and 483.2 mg/L of 3′-SL, respectively. The study concluded that concentrations up to 500 mg/L are safe and supported similar growth and development of piglets fed the control diet.

Wang et al. (2019) performed a study using sow replacement milk supplemented with a combination of 7.6 g/kg 3'-SL and1.9 g/kg 6'-SL to observe the effect that sialylated milk oligosaccharides had on neurotransmitters and brain metabolites in piglets. Neonatal piglets were fed sow replacement milk supplemented with sialylated oligosaccharides from 3 days to 38 days of age. The sialylated oligosaccharide intervention did not significantly influence body weight gain, brain weight gain, or weight gain in specific regions of the brain compared to controls.

Obelitz-Ryom et al. (2019) fed preterm piglets either intact unpasteurized Jersey cow's milk supplemented with lactose, intact unpasteurized Jersey cow's milk supplemented with 4.5% sialyllactose (a 6:1 ratio of 3'-SL and 6'-SL), or their mother's milk only for 19 days. In addition to assessing cognitive performance, clinical outcomes and growth were evaluated. Although the growth of the pigs receiving the cow's milk supplemented with either lactose or sialyllactoses

was less than the pig receiving their mother's milk, as judged by weight gain, no adverse events related to the experimental diet were reported in the study.

G. CLINICAL STUDIES

Seven published clinical studies have evaluated the tolerability of 3'-SL alone or in combination with 6'-SL (Cooper et al., 2017; Meli et al., 2014; Radke et al., 2017; Simeoni et al., 2016; Parente et al., 2003; Rasko et al., 2000; Gurung et al., 2018). Four of these clinical trials were conducted among infants where term infant formulas supplemented with bovine milk-derived oligosaccharides (BMOS) and unspecified amounts of 3'-SL and 6'-SL were administered (Cooper et al., 2017; Meli et al., 2014; Radke et al., 2017; Simeoni et al., 2016). The remaining three published clinical studies were conducted in adults with *H. pylori* infections (Parente et al., 2003; Rasko et al., 2000; Gurung et al., 2018). Collectively, all of the clinical studies show that term infant formulas supplemented with 3'-SL and 6'-SL do not affect normal growth and development in infants and 3'-SL supplementation up to 20 g/day is well tolerated in adults.

1. Infants

A literature search conducted through February 24, 2020 revealed that the only clinical studies conducted in infants are those that have tested the safety/tolerability of infant formulas supplemented with bovine milk-derived oligosaccharides (BMOS) and unspecified amounts of 3'-SL and 6'-SL (Cooper et al., 2017; Meli et al., 2014; Radke et al., 2017; Simeoni et al., 2016). Because these studies were summarized in GRN 766 (pg. 62-64), but not GRN 880, the summaries provided in GRN 766 are therefore incorporated by reference and briefly summarized in Table 28. All studies concluded that compared to standard formula without HMOs, term infant formulas supplemented with BMOS, 3'-SL, and 6'-SL were well-tolerated and no effect on growth and development.

2. Adults

A literature search conducted through February 24, 2020 revealed that no new clinical trials investigating the tolerability of 3'-SL intake in adults have been published since the filing of GRN 880. Therefore, the two studies summarized in GRN 766 (pg. 64-67) and the one study summarized in GRN 880 (pg. 35, 36) are incorporated by reference and briefly summarized in Table 28 (Parente et al., 2003; Rasko et al., 2000; Gurung et al., 2018). In summary, doses up to 20 g/day 3'-SL sodium salt were administered to patients with *H. pylori* infections were well tolerated.

Table 28. Clinical Studies of 3'-Sialyllactose

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters
Infant Studies				
Meli et al. 2014	Randomized, double-blind, single-center study on healthy term infants (<14 days old)	Control formula, n=84 Standard formula plus 10 g/L BMOS (bovine MOS including GOS and 3' and 6'-SL (concentrations unknown)), n= 99 Standard formula plus 10 g/L BMOS and the probiotics *Bifidobacterium longum* ATCC BAA-999 (Bl999) and Lactobacillus rhamnosus CGMCC 1.3724 (LPR) each at 2×10^7 cfu/g, n=98 Breastfed reference group, n=39	4 months	• 90 infants from formula groups and 18 infants from breastfed groups withdrew • Weight gain and length and head circumference showed no significant differences between control and BMOS formula groups • BMOS groups had more frequent and less hard stools compared to control • No significant differences were observed between the control and BMOS groups in caregivers' reports of flatulence, vomiting, spitting up, crying, fussing, and colic.
Simeoni et al. 2016	Randomized, placebo-controlled, double-blind study on healthy term newborns	Control formula, n=37 Test formula group, standard formula plus 5.7±1.0 g/100 g bovine milk oligosaccharides (BMOS which includes 3'-SL) and $1x10^7$ cfu/g of *B. lactis* CNCM I-3446, n=39 Reference group: breastfed, n=37	12 weeks	• No difference in compliance or tolerability was observed between the three groups. • No difference was seen in anthropometric measures between the three groups. • The BMOS supplemented formula had no adverse effects on fecal microbiota.

Table 28. Clinical Studies of 3'-Sialyllactose

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters
Cooper et al. 2017	Multicenter, randomized, placebo-controlled, double-blind study on healthy infants born to HIV+ mothers in South Africa	Cesarean-delivered infants control formula, n=101 Cesarean-delivered infants starter formula containing 5.8 ± 1.0 g/100 g of powder formula (8 g/L in the reconstituted formula) BMOS and $1x10^7$ cfu/g *B. lactis* CNCM I-3446, n=92 Vaginally delivered infants control formula, n=113 Vaginally delivered infants starter formula containing 5.8 ± 1.0 g/100 g of powder formula [8 g/L in the reconstituted formula BMOS and $1x10^7$ cfu/g *B. lactis* CNCM I-3446, n=115	4 months	• No difference was seen in anthropometric measures between the two groups in both delivery methods. • No significant difference was observed in tolerability and adverse events between the groups in both delivery methods. • Test formula supplemented with BMOS lowered fecal pH and improved fecal microbiota counts in both delivery methods.
Radke et al. 2017	Multicenter, randomized placebo-controlled, double-blind study on healthy infants in Germany	Control formula, n=207 Test formula containing 5.8 ± 1.0 g/100 g of powder formula (8 g/L in the reconstituted formula) BMOS (containing GOS and 3'-SL) and $1x10^7$ cfu/g *B. lactis* CNCM I-3446, n=206 Breastfed reference group, n=63	6 months Follow-up at 12 months, no test formula 6-12 months	• No significant difference in diarrhea or febrile infections incidence between the groups at 6 and 12 months. • Test formula was well tolerated and no difference in anthropometric measures were observed between the groups. • The test formula group showed similar gut microbiota patterns, fecal IgA, and stool pH to breastfed infants and was significantly different than the control formula group.

Table 28. Clinical Studies of 3'-Sialyllactose

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters
Adult Studies				
Rasko et al. 2000	Randomized, double-blind, placebo-controlled study on adults with *H. pylori* infection	Control group (placebo), n=6 Group 1: 4g 3'-SL, n=6 Group 2: 8g 3'-SL, n=7 Group 3: 20g 3'-SL, n=7	56 days for Control and Groups 1 and 2 28 days for Group 3	• Oral supplementation of 3'-SL did not change Lewis antigen expression of *H. pylori* strains isolated from human gastric mucosa. • No adverse effects on safety or tolerance were reported.
Parente et al. 2003	Randomized, double-blind, placebo-controlled study on adults with *H. pylori* infection (dyspepsia)	Control group (placebo), n=21 Group 1: 10 g/day of 3'-SL sodium salt, n=17 Group 2: 20 g/day of 3'-SL sodium salt, n=22	4 weeks	• Five patients were excluded from analysis due to protocol violation. • Adverse events recorded in 6 patients were halitosis, asthenia, epigastric pain, and headache. One patient dropped out due to headache associated with epigastric pain. No serious adverse events were observed. • *H. pylori* colonization documented by the ^{13}C-Urea Breath Test (UBT) decreased significantly (*p*-value not provided) in both treatment groups and placebo but was most likely due to regression toward mean effect.
Gurung et al. 2018	Randomized, double-blind, placebo-controlled study on adults with *H. pylori* infection	Control group (placebo): n=17 Group 1: 12 g/day, n=24	4 weeks	• There were no significant differences between pre- and post-dose gastrointestinal tolerance and clinical chemistry (serum biochemistry, hematology, and urine analysis) outcomes. • Pre- and post-dose urea breath test values were not significantly different within or between the 3'-SL and placebo groups. • Compliance and adverse events were similar between the groups.

H. ALLERGENICITY

Allergens, by definition, are antigens that are recognized by IgE antibodies and provoke IgE-mediated hypersensitivity responses (Aalberse, 2000). Most allergens are proteins or glycoproteins (Radauer et al., 2008; Sicherer and Sampson, 1999), although there have been a limited number of reports of allergic reactions to carbohydrates (Franck et al., 2005; Chiang et al., 2012; Commins et al., 2009). Additionally, allergic reactions to human milk have not been reported. Importantly, genetically engineered strains of *E. coli* BL21(DE3) have been safely used in the production of food and pharmaceutical ingredients (see Section VI.A) and product specifications control the level of protein derived from *JBT-3SL* in the finished ingredient (see Section II.G).Moreover, the genes used to engineer *JBT-3SL* are not derived from major allergens and full-length FASTA alignments of amino acid sequences of the genes used to engineer JBT-3SL and version 19 of the AllergenOnline Database maintained by the University of Nebraska – Lincoln showed that cross-reactivity with known allergens (≥ 50% identity) is not expected (Table 29). Thus, although the protein specification does not eliminate the possibility that consumers of Jennewein's 3'-SL-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 *JBT-3SL* in the finished ingredient are not expected.

Table 29. Percent Identity of the Genetic Manipulations in *JBT-3SL* with Known Allergens

Function	Origin of the gene	% Identity*
Lactose permease	*E. coli* K12	27.4
UDP-galactose-4-epimerase	*E. coli* K12	30.7
Galactokinase	*E. coli* K12	None
Galactose mutarotase	*E. coli* K12	26.9
Galactosyltransferase	*E. coli* K12	24.0
Glutamine fructose 6-phosphate aminotransferase	*E. coli* K12	23.2
Glucosamine 6-phosphate N-acetyltransferase	*Saccharomyces cerevisiae*	None
N-Acetylglucosamine 2-epimerase	*Synechocystis sp.* PCC6803	≤ 25.9
N-Acetylneuraminic acid synthetase	*Campylobacter jejuni*	≤ 27.8
CMP *N*-acetylneuraminic acid synthase	*Campylobacter jejuni*	None
α 2,3-sialylltransferase	*Heamophilus parahaemolyticus*	26.1
Antibiotic Resistance Genes		
Dihydrofolate reductase conferring resistance to trimethoprim	*Citrobacter freundii*	≤ 34.9
Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	None
Neomycin phosphotransferase II conferring resistance to kanamycin	*Tn5 E. coli* K12	None
Gentamycin 3'-acetyltransferase conferring resistance to gentamycin	*Acinetobacter baumannii* AYE	None

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

I. REGULATORY APPROVALS AROUND THE WORLD

3'-Sialyllactose is GRAS in the United States and is the subject of two GRAS notifications (GRN 766 and 880). GeneChem's 3'-SL, produced by an enzymatic reaction, received a "no questions" letter from the FDA and is intended for use in non-exempt infant formula at a maximum use level of 0.238 g/L and other foods and beverages at a maximum use level of 24-3000 mg/serving. Glycom's 3'-SL GRN 880, produced by fermentation similar to Jennewein, is still pending a decision from the FDA, but is intended for use in infant formula at a maximum use level of 0.2 g/L, 0.15 g/L in follow-on formula and infant-specific beverages, up to 2.5 g/kg in other foods and beverages, and 5 g/kg in foods for special dietary use. It is also the subject of a Novel Food application in the European Union (https://ec.europa.eu/food/sites/food/files/safety/docs/novel-food_sum_ongoing-app_2019-0881.pdf; accessed on February 24, 2020), although opinions by the European Food Safety Authority (EFSA) and/or the European Commission have not been published.

VII. SUPPORTING DATA AND INFORMATION

A. REFERENCES

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

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B. EXPERT PANEL STATEMENT

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of 3'-sialyllactose sodium salt (3'-SL) in non-exempt term infant formula has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). The safety of the intake of 3'-SL in non-exempt term infant formula has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food and is based on generally available and accepted information.

The use of 3'-SL as an ingredient for the intended use in infant formula has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. The subject of this GRAS Notice is a spray-dried, powdered food ingredient that contains not less than 88% 3'-SL dry weight.

    a. 3'-Sialyllactose is a naturally occurring acidic oligosaccharide in human milk.

    b. The 3'-SL that is the subject of this GRAS Notice is structurally identical to the 3'-SL present in human breast milk.

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

    d. The subject of this GRAS Notice is manufactured using a genetically engineered strain of *Escherichia coli* BL21(DE3). Because the host strain does not possess the components required for *E. coli* pathogenicity, strains derived from *E. coli* BL21(DE3) from it are suitable for the production of food ingredients.

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

f. Fermentation by-products include lactose, sialic acid, and *N*-acetylglucosamine which are known human milk oligosaccharides; their presence in the finished ingredient is not of toxicological concern.

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

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

2. Human milk oligosaccharides, including 3'-SL, are resistant to the digestive enzymes in the gastrointestinal tract, poorly absorbed, and pass through the gastrointestinal tract where they are either fermented by the microbiota or excreted unchanged.

3. Published studies showing that the amount of 3'-SL in breast milk ranges from 0.08 to 0.41 g/L.

4. Genotoxicology and subchronic toxicology studies published by Phipps et al. (2019) show that 3'-SL is not genotoxic and has a NOAEL (no observed adverse effect level) of 5 g/kg bw/day, which was the highest dose tested.

5. The addition of 0.28 g/L 3'-SL in infant formula will result in mean and 90th percentile estimated daily intakes (EDI) of 0.278 g/day (43.1 mg/kg bw/day) and 0.325 g/day (50.4 mg/kg bw/day) for infants 0 to 12 months-of-age.

6. The safety of exposure to Jennewein's 3'-SL at its intended use level is supported by:

a. Published studies that quantitate the levels of 3'-SL in human milk;

b. Analytical data demonstrating that the 3'-SL produced by Jennewein is structurally identical to 3'-SL from human milk;

c. Data demonstrating the qualitative and quantitative similarities between the subject of this GRAS Notice and the 3'-SL ingredient tested by Phipps et al. (2019), which is the subject of GRN 880;

d. Corroborative published genotoxicology and 90-day subchronic toxicology studies conducted with 3'-SL or a mixture of human milk oligosaccharides containing 4.1 % of Jennewein-manufactured 3'-SL.

7. A corroborative unpublished tolerance study in neonatal piglets conducted with a mixture of HMOs containing up to 0.28 g/L of Jennewein's 3'-SL ingredient that showed an HMO mixture containing 3'-SL was well-tolerated and supported normal growth in neonatal piglets.

Therefore, 3'-SL is safe and GRAS at the proposed level of addition to the intended non-exempt, term infant formula. 3'-Sialyllactose is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

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

Signature: [redacted]

Date: March 19, 2020

A. Wallace Hayes, PhD, DABT, FATS, ERT
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Thomas E. Sox, PhD, JD
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Date: March 19, 2020

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel

Signatur [redacted]

Date: March 19, 2020

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000921	Mar 23, 2020
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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

1. Type of Submission *(Check one)*

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

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

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

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

Name of Contact Person: Julia Parkot		Position or Title: Head of Quality Unit and Regulatory Affairs	
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Mailing Address *(number and street)*: Maarweg 32			
City: Rheinbreitbach	State or Province: Rheinbreitbach	Zip Code/Postal Code: D-53619	Country: Germany
Telephone Number: +49 - (0)2224-98810-251	Fax Number:	E-Mail Address: julia.parkot@jennewein-biotech.de	

Name of Contact Person: Dietrich B. Conze, PhD		Position or Title: Managing Partner	
Organization *(if applicable)*: Spherix Consulting Group, Inc.			
Mailing Address *(number and street)*: 11821 Parklawn Drive, Suite 310			
City: Rockville	State or Province: Maryland	Zip Code/Postal Code: 20852	Country: United States of America
Telephone Number: 240-367-6089	Fax Number:	E-Mail Address: dconze@spherixgroup.com	

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

3'-Sialyllactose sodium salt (3'-SL)

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

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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

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

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

☒ a) GRAS Notice No. GRN 571

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRNs 766, 880

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

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 intends to use 3'-SL sodium salt as a substitute for other forms of 3'-SL in powdered, cow's milk non-exempt infant formula for term infants at a level of 0.27 g/L.

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

1. The undersigned is informing FDA that Jennewein Biotechnologie GmbH

(name of notifier)

has concluded that the intended use*(s)* of 3'-Sialyllactose sodium salt (3'-SL)

(name of notified substance)

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

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

(name of notifier)

Maarweg 32, D-53619 Rheinbreitbach, Germany

(address of notifier or other location)

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Dietrich B. Conze, PhD Digitally signed by Dietrich B. Conze, PhD Date: 2020.03.20 12:43:23 -04'00'	Dietrich B. Conze, PhD, Managing Partner	03/19/2020

SECTION G – LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Jennewein 3'-SL GRAS Final to FDA.pdf	Submission
	References	Submission

From:	kbrailer@spherixgroup.com
To:	Morissette, Rachel
Cc:	Honigfort, Mical; Morissette, Rachel; "Claire Kruger"; "Dietrich Conze"; West-Barnette, Shayla
Subject:	RE: GRN 000921 questions
Date:	Friday, July 31, 2020 5:37:06 PM
Attachments:	Response to FDA Questions on GRN921 7-31-2020.pdf

Dear Dr. Morissette:

Attached please find our response to your request for additional information regarding GRN 000921. Please confirm receipt and let us know if you need any additional information.

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: Dietrich Conze <dconze@spherixgroup.com>
Sent: Monday, July 13, 2020 2:24 PM
To: West-Barnette, Shayla <Shayla.WestBarnette@fda.hhs.gov>
Cc: Honigfort, Mical <Mical.Honigfort@fda.hhs.gov>; Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>; Claire Kruger <ckruger@spherixgroup.com>; Kathy Brailer <kbrailer@spherixgroup.com>
Subject: Re: GRN 000921 questions

Thank you Shayla.
Dietz

> On Jul 13, 2020, at 2:22 PM, West-Barnette, Shayla <Shayla.WestBarnette@fda.hhs.gov> wrote:
>
> Hello Dr. Conze,
>
> Thank you for contacting us. An additional five days to respond to our questions for GRN 921 would be fine. We look forward to received your responses by cob July 31, 2020.
>
> Regards,
>
> Shayla West-Barnette, Ph.D.

From: Dietrich Conze <dconze@spherixgroup.com>
Sent: Monday, July 13, 2020 2:17 PM
To: West-Barnette, Shayla <Shayla.WestBarnette@fda.hhs.gov>; Honigfort, Mical <Mical.Honigfort@fda.hhs.gov>
Cc: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>; Claire Kruger <ckruger@spherixgroup.com>; Kathy Brailer <kbrailer@spherixgroup.com>
Subject: GRN 000921 questions

Hi Shayla and Mical,

I received the out of office notice from Rachel Morissette (below) regarding our responses to the questions for GRN 000921, which were received on Friday, July 10th. I have to coordinate our responses with our client and am wondering if it would be possible for us to have an additional 5 days to draft our responses. If so, we would be sending you our response no later than July 31, 2020.

Regards.
Dietrich

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 Jul 13, 2020, at 2:04 PM, Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:
>
> I am out of the office and will return on July 20. For urgent matters, please contact Dr. Shayla West-Barnette (shayla.westbarnette@fda.hhs.gov) and/or Dr. Mical Honigfort (mical.honigfort@fda.hhs.gov).

Spherix Consulting Group

July 31, 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 000921

Dear Dr. Morissette:

In response to your email of July 10, 2020, below are our responses to your request for additional information regarding GRN 000921. FDA's questions are italicized text and our responses are in plain text.

Regulatory:

1. *Please provide a statement that Jennewein concludes that the intended use of 3'-SL is GRAS.*

   Based on the totality of available safety data, there is reasonable certainty that Jennewein's 3'-SL is of no harm to consumers per the intended use and use level. Jennewein therefore concludes that 3'-SL is GRAS as an ingredient in term, non-exempt infant formula at the intended use level.

2. *On page 19 of the notice under C. Intended Uses, Jennewein refers to GRN 776. Please clarify if GRN 000766 is intended here.*

   Yes, Jennewein intends to refer to GRN 000766 on page 19 of the Notice under C. Intended Uses.

Chemistry:

3. *On page 5 of the notice, the CAS registry number cited (37449-93-7) is for 3'-sialyllactose. However, the subject of the notice is the sodium salt of 3'-sialyllactose, which has the CAS registry number 128596-80-5. Please clarify this discrepancy.*

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http://spherixgroup.html

Because the subject of this Notice is 3′-sialyllactose sodium salt, the CAS registry number 37449-93-7 cited in the original Notice is wrong. The correct CAS registry number is 128596-08-5.

Microbiology:

4. Please state whether Escherichia coli BL21(DE3) strain "JBT-3SL" has been deposited in a recognized culture collection and provide the non-trade name designation. If the strain is not deposited, please describe how the source was verified and identified.

JBT-3SL has been deposited at DMSZ - German Collection of Microorganisms and Cell Cultures GmbH with the following deposition number DSM 33492.

5. Please state whether E. coli BL21(DE3) strain "JBT-3SL" is non-pathogenic and non-toxigenic.

JBT-3SL is non-pathogenic and non-toxigenic.

6. Jennewein states that E. coli BL21(DE3) has an absence of genes encoding invasion factors, adhesion molecules, and enterotoxins associated with virulence. Please state whether E. coli BL21(DE3) strain "JBT-3SL" has the same virulence profile.

Jennewein engineered JBT-3SL using site-specific homologous recombination or transposition of genes with known function that do not confer virulence. JBT-3SL also does not harbor any additional plasmids. Therefore, JBT-3SL has the same virulence profile as *E. coli* BL21(DE3).

7. Jennewein states that the use of E. coli BL21(DE3) is not expected to result in any safety concerns. Please state whether E. coli BL21(DE3) strain "JBT-3SL" is expected to result in any safety concerns.

Because Jennewein engineered the strain JBT-3SL by inserting genes with known functions that are not associated with virulence and pathogenicity, and the strain harbors no additional plasmids, JBT-3SL is not expected to result in any safety concerns.

8. Please state whether E. coli BL21(DE3) strain "JBT-3SL" is capable of DNA transfer to other organisms.

Because *E. coli* BL21(DE3) is not able to transfer DNA to other organisms and Jennewein inserted only genetic elements that do not confer the ability to transfer DNA to other organisms during the engineering of *E. coli* BL21(DE3) strain JBT-3SL, JBT-3SL is not capable of DNA transfer to other organisms.

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

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

Toxicology:

10. *In Table 7 on page 18 of the notice, Jennewein provides data on the concentrations of 3'-SL in human breast milk. Jennewein cites Thurl et al. (2017) and provides data from eight additional studies to provide a basis for the proposed use level of 0.28 g/L. We note that the proposed use level is higher than the mean and the value for the upper 95% confidence interval for term deliveries as reported by Thurl et al. Given that Thurl et al. conducted a systematic review and reported robust inclusion and exclusion criteria for the selection of studies, please provide a clear rationale as to how the additional studies cited by Jennewein meet the criteria for comparison to the studies in the systematic review by Thurl et al. and discuss how these findings justify a weight-of-evidence shift that would support the proposed higher use level.*

    The inclusion criteria employed by Thurl et al. (2017) were: milk samples from individual healthy mothers; documented duration of pregnancy; documented lactation days; individuals with defined secretor status in the case of neutral HMOs; absolute concentrations of single HMOs; mean values, single HMO concentrations values with an n of not less than two at a given gestational age, secretor status and lactation period (i.e., values from at least two mothers were required); and original articles from peer-reviewed journals. The exclusion criteria were: animal studies; pooled milk samples; milk samples at lactation periods not fitting the lactation periods defined in the review; relative concentrations; concentration of mixtures of HMOs; median values; mass spectrometry data based on universal calibration; abstracts; monographs; review articles; and studies with all data already reported.

    Although Kunz et al. (2017) reported a median concentration for 3'-SL in human milk, all of the remaining studies included in Table 7 meet the inclusion criteria used by Thurl et al. and are therefore, of similar quality to those reviewed and evaluated by Thurl et al. Additionally, all of the studies included in Table 7 were also used to support the intended use level of the 3'-SL that is the subject of GRN 000880. Thus, the studies included in Table 7 must be considered when evaluating the weight-of-the-evidence to establish the level of 3'-SL in human milk and support the proposed intended use level of Jennewein's 3'-SL in infant formula.

11. *On page 26 of the notice, Jennewein notes that sialic acid is a major degradation product of 3'-SL. Additionally, in Table 8 on page 26, Jennewein's specifications for sialic acid are significantly higher than the specifications for the 3'-SL in GRN 000880, to which the notified ingredient is being compared. Please discuss why the sialic acid cleaved from 3'-SL via microbial and intestinal neuraminidases combined with the sialic acid present as a byproduct in the ingredient is not a safety concern.*

    Free sialic acid is a component of human milk, with levels ranging from 900 to 1800 mg/L in colostrum, transitional milk and first month milk, and from 300 to 800 mg/L in mature milk (Hayakawa et al., 1993; Wang et al., 2001; Martin-Sosa et al., 2004; Oriquat et al., 2011; Wang 2001; Martin-Sosa et al., 2004; Qiao et al., 2013). Free sialic acid is also GRAS for use in infant formula at 50 mg/L (GRN 000602). Although sialic acid is a

major degradation product of 3'-SL and the specifications for sialic acid are significantly higher than the specifications for the 3'-SL in GRN 000880, it is important to note that Jennewein's sialic acid specification exceeds the actual amount of sialic acid in the product by approximately 23-fold. The actual levels of sialic acid are approximately 0.425% on a percent area basis (see Table 3 in Notice). On a dry matter basis, sialic acid is present at approximately 0.275% (see Table below).

Assuming that infants consume one liter of 3'-SL-containing infant formula/day, the resulting intake of the free sialic acid impurity, based on actual levels, will be 0.77 mg/day on a dry matter basis (0.275% multiplied by the intended use level of 0.28 g 3'-SL/L). If sialic acid were present at the specification, the maximum intake of the impurity would be approximately 17.7 mg/day, 23-fold higher than the actual levels. Importantly, both the actual and maximum sialic acid impurity intakes are orders of magnitude below the ingestion of free sialic acid from human milk and the levels that have been determined GRAS, as described above.

Because the intended use level of 3'-SL is based on the levels of 3'-SL reported in human milk (see Section III.B of the GRAS Notice) and the ingestion of the sialic acid impurity is below the reported intakes from human milk, the ingestion of sialic acid cleaved from 3'-SL via microbial and intestinal neuraminidases combined with the sialic acid present as a byproduct in the ingredient is equivalent to what is ingested with human milk and thus not a safety concern.

Table 1. Actual Levels of Sialic Acid in Jennewein 3'-SL[1]

Lot Number	Sialic Acid (% DM)
11027019	0.2 %
11030019	0.2 %
11030029	0.3 %
11031039	0.4 %
Average	0.275%

Abbreviations: DM – dry matter
[1]Determined high performance anion exchange chromatography coupled with pulsed amperometric detection with a calibration adjusted to the expected range for sialic acid.

References

Hayakawa, K., De Felice, C., Watanabe, T., Tanaka, T., Iinuma, K., Nihei, K., Higuchi, S., Ezoe, T., Hibi, I., and Kurosawa, K. (1993). Determination of free N-acetylneuraminic acid in human body fluids by high-performance liquid chromatography with fluorimetric detection. J Chromatogr 620, 25–31.

Martín-Sosa, S., Martín, M.J., García-Pardo, L.A., and Hueso, P. (2004). Distribution of sialic acids in the milk of spanish mothers of full-term infants during lactation. J Pediatr Gastroenterol Nutr 39, 499–503.

Oriquat, G.A., Saleem, T.H., Abdullah, S.T., Soliman, G.T., Yousef, R.S., Hameed, A.M.A., and Salim, M.L (2011). Soluble CD14, sialic acid, and I-fucose in breast milk and their role in increasing the immunity of breast-fed infants. Am J Biochem. Biotech. 7, 21–28.

Qiao, Y., Feng, J., Yang, J., and Gu, G. (2013). The relationship between dietary vitamin A intake and the levels of sialic acid in the breast milk of lactating women. J Nutr Sci Vitaminol (Tokyo) 59, 347–351.

Thurl, S., Muller-Werner, B., and Sawatzki, G. (1996). Quantification of individual oligosaccharide compounds from human milk using high-pH anion-exchange chromatography. Anal Biochem 235, 202–206.

Wang, B., Brand-Miller, J., McVeagh, P., and Petocz, P. (2001). Concentration and distribution of sialic acid in human milk and infant formulas. Am J Clin Nutr 74, 510–515.

Wiederschain, G.Y., and Newburg, D.S. (2001). Glycoconjugate stability in human milk: glycosidase activities and sugar release. J Nutr Biochem 12, 559–564.

12. *There are conflicting reports in the literature regarding the ability of 3'-SL to activate the Toll-like receptor 4 (TLR4) signaling pathway[1,2], which were not reviewed in the notice. Please discuss if Jennewein agrees or disagrees that 3'-SL is an activator of the TLR4 signaling pathway and if the information in the references cited herein impacts the overall safety assessment of 3'-SL.*

Toll-like receptor 4 (TLR4) specifically recognizes lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, and its activation leads to the synthesis and production of pro-inflammatory cytokines and chemokines (Janssens et al., 2003). Other TLR4 ligands have been identified such as mannuronic acid polymers from Gram-negative bacteria, teichuronic acid from Gram-positive bacteria, and viral components such as the F-protein of respiratory syncytial virus (reviewed in Vaure and Liu, 2014). Additionally, it is well known that intravenous administration of LPS induces severe toxicity whereas the ingestion LPS is not associated with adverse effects, which is consistent with the fact that humans are exposed to microbiota-derived LPS in their gastrointestinal tracts and do not develop septic shock (reviewed in Wassenaar and Zimmermann, 2018).

Although the *in vitro* studies conducted by Kurakevich et al. suggest that 3'-SL may be a TLR4 agonist, it is not possible to rule out the fact that contaminating LPS in the 3'-SL preparation may have been responsible for the TLR4-induced effects in light of the results reported by Perdijk et al. (2019). Additionally, other than stating that the 3'-SL product was chemically synthesized and LPS was undetectable via a limulus assay (limit of detection of 0.05 endotoxin unit/ml), Kurakevich et al. did not provide specific details on how the 3'-SL was manufactured or evaluate whether 3'-SL products manufactured by

other manufacturers had similar effects, and a recent study has shown that the administration of 3'-SL manufactured using a genetically modified strain of *Escherichia coli* does not aggravate the spontaneous inflammation that develops in the gastrointestinal tract of mice deficient in the anti-inflammatory cytokine interleukin 10 (Grabinger et al., 2019). Thus, considering the currently available public data, 3'-SL does not appear to be a TLR4 agonist.

Importantly, the toxicity of 3'-SL has been rigorously evaluated in an acute single dose toxicity study in rats, a 28-day sub-chronic oral toxicity study in rats, two sub-chronic 90-day oral toxicity studies in rats, and an acute single escalating-dose oral toxicity study in Beagle dogs at doses up to 5 g/kg bw/day (Kim et al., 2018; Phipps et al., 2019). No treatment-related adverse effects were reported. Moreover, the intended use levels of 3'-SL mimic those found in human milk. Thus, the potential effect of 3'-SL on TLR4 signaling pathways or lack thereof will be comparable to those occurring in breast-fed infants consuming 3'-SL-containing human milk and does not impact the overall safety assessment of 3′-SL.

References

Grabinger, T., Glaus Garzon, J.F., Hausmann, M., Geirnaert, A., Lacroix, C., and Hennet, T. (2019). Alleviation of Intestinal Inflammation by Oral Supplementation With 2-Fucosyllactose in Mice. Front Microbiol 10, 1385.

Janssens, S., and Beyaert, R. (2003). Role of Toll-like receptors in pathogen recognition. Clin Microbiol Rev 16, 637–646.

Kim, D., Gurung, R.B., Seo, W., Lee, A.W., and Woo, J. (2018). Toxicological evaluation of 3'-sialyllactose sodium salt. Regul Toxicol Pharmacol 94, 83–90.

Perdijk, O., van Neerven, R.J.J., Meijer, B., Savelkoul, H.F.J., and Brugman, S. (2018). Induction of human tolerogenic dendritic cells by 3'-sialyllactose via TLR4 is explained by LPS contamination. Glycobiology 28, 126–130.

Phipps, K.R., Baldwin, N.J., Lynch, B., Stannard, D.R., Šoltésová, A., Gilby, B., Mikš, M.H., and Röhrig, C.H. (2019). Toxicological safety assessment of the human-identical milk oligosaccharide 3'-sialyllactose sodium salt. J Appl Toxicol.

Vaure, C., and Liu, Y. (2014). A comparative review of toll-like receptor 4 expression and functionality in different animal species. Front Immunol *5*, 316.

Wassenaar, T.M., and Zimmermann, K. (2018). Lipopolysaccharides in Food, Food Supplements, and Probiotics: Should We be Worried. Eur J Microbiol Immunol (Bp) *8*, 63–69.

13. In Part VI.B. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION on page 25 of the notice, FDA notes that several references cited are not included in the references section, including: Ruhaak et al., 2014; Santos-Fandila et al., 2014; Dotz et al., 2014; Obermeier et al., 1999; Rudloff et al., 2012; Rudloff et al., 2006; Rudloff and Kunz, 2012; Rudloff et al., 1996; Chaturvedi et al., 2001; Engfer et al., 2000; and Brand-Miller et al., 1998. Please provide an updated list that includes these references.

An updated list of references, which includes those that were not included in the original notification, is provided below.

Aakko J, Kumar H, Rautava S, Wise A, Autran C, Bode L, et al. (2017). Human milk oligosaccharide categories define the microbiota composition in human colostrum. Benef Microbes 8(4):563-567.

Aalberse, R.C. (2000). Structural biology of allergens. J Allergy Clin Immunol 106, 228–238.

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.

Ames BN, Durston WE, Yamasaki E, d. Lee F. (1973) Carcinogens are Mutagens: A Simple Test System Combining Liver Homogenates for Activation and Bacteria for Detection. Proc. Natl. Acad. Sci. U. S. A. 70: 2281–2285.

Ames BN, McCann J, Yamasaki E. (1975) Methods for detecting carcinogens and mutagens with the salmonella/mammalian-microsome mutagenicity test. Mutation Research/Environmental Mutagenesis and Related Subjects. 31: 347–363.

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, Akahori M, Kimura K, Watanabe Y, Nakamura T, Tsunemi M, Arai I, Sanai Y, Urashima T (2007). Sialyl oligosaccharides of human colostrum: changes in concentration during the first three days of lactation. Biosci Biotech Biochem 71:1447-1451.

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

Azad MB, Robertson B, Atakora F, Becker AB, Subbarao P, Moraes TJ, et al. (2018). Human milk oligosaccharide concentrations are associated with multiple fixed and modifiable maternal characteristics, environmental factors, and feeding practices. J Nutr 148:1-10.

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Berger, P.K., Plows, J.F., Jones, R.B., Alderete, T.L., Yonemitsu, C., Poulsen, M., Ryoo, J.H., Peterson, B.S., Bode, L., and Goran, M.I. (2020). Human milk oligosaccharide 2'-fucosyllactose links feedings at 1 month to cognitive development at 24 months in infants of normal and overweight mothers. PLoS One 15, e0228323.

Bode L (2012a). Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 22(9):1147-1162.

Bode, L., and Jantscher-Krenn, E. (2012b). Structure-Function Relationships of Human Milk Oligosaccharides. Advances in Nutrition 3, 383S–391S.

Brand-Miller, J.C., McVeagh, P., McNeil, Y., and Messer, M. (1998). Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. J Pediatr 133, 95–98.

Burgeiro, A., Cerqueira, M.G., Varela-Rodríguez, B.M., Nunes, S., Neto, P., Pereira, F.C., Reis, F., and Carvalho, E. (2017). Glucose and Lipid Dysmetabolism in a Rat Model of Prediabetes Induced by a High-Sucrose Diet. Nutrients 9, 638.

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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

From:	Dietrich Conze
To:	Morissette, Rachel
Cc:	Kathy Brailer; Claire Kruger
Subject:	Re: clarification questions for GRN 000921
Date:	Monday, October 12, 2020 6:57:17 PM
Attachments:	Response to FDA Questions on GRN921 10-12-2020.pdf

Hi Rachel,
I apologize for the delay. Attached are our responses to the 2nd round of questions for GRN 000921. If you have any additional questions, please let me know.
Regards.
Dietz

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> On Sep 15, 2020, at 2:40 PM, Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:
>
> Thank you.
>
> *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:** Tuesday, September 15, 2020 2:32 PM
> **To:** Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
> **Cc:** Kathy Brailer <kbrailer@spherixgroup.com>; Claire Kruger <ckruger@spherixgroup.com>
> **Subject:** Re: clarification questions for GRN 000921

Hi Rachel,
Thank you for the email. We are coordinating with our client and will be in touch shortly.
Regards.
Dietz

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> On Sep 15, 2020, at 8:50 AM, Morissette, Rachel <Rachel.Morissette@fda.hhs.gov> wrote:
>
> Dear Dr. Conze,
>
> We had a few clarification questions regarding your July 31, 2020 amendment.
>
> 1. In the response to question 10, Jennewein states, "Additionally, all of the studies included in Table 7 were also used to support the intended use level of the 3′-SL that is the subject of GRN 000880." We note that the notifier for GRN 000880 intended a lower use level based on their own analysis of the 3′-SL levels reported in these studies; thus, it is not clear in Jennewein's response how they arrived at a different and higher use level. Specifically, the notifier for GRN 000880 stratified these studies by period of lactation and calculated an average value for each lactation time. We note that in "days 10-60 (mature milk)," the calculated average is 0.27 g/L, which is very close to the intended use level in the current notice and may potentially serve as a basis for Jennewein's intended use level. Given that 3′-SL levels are reported to be relatively stable throughout lactation, there may be an additional justification for Jennewein's intended use level based on the published literature. Please provide any additional rationale, including any specific

published data and data analysis to support the intended higher use level of 3′-SL.

2. In the response to our questions, Jennewein states that *E. coli* BL21(DE3) strain “JBT-3SL” is deposited in the “DMSZ – German Collection of Microorganisms and Cell Cultures GmbH”. Please clarify if the deposit designation is supposed to be “DSMZ – German Collection of Microorganisms and Cell Cultures GmbH”?

Thank you for your attention to this matter.

Best regards,

Rachel

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Spherix Consulting Group

October 12, 2020

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RE: Questions Regarding GRN 000921

Dear Dr. Morissette:

In response to your email of September 15, 2020, below are our responses to your request for additional information regarding GRN 000921. FDA's questions are italicized text and our responses are in plain text.

1. *In the response to question 10, Jennewein states, "Additionally, all of the studies included in Table 7 were also used to support the intended use level of the 3'-SL that is the subject of GRN 000880." We note that the notifier for GRN 000880 intended a lower use level based on their own analysis of the 3'-SL levels reported in these studies; thus, it is not clear in Jennewein's response how they arrived at a different and higher use level. Specifically, the notifier for GRN 000880 stratified these studies by period of lactation and calculated an average value for each lactation time. We note that in "days 10-60 (mature milk)," the calculated average is 0.27 g/L, which is very close to the intended use level in the current notice and may potentially serve as a basis for Jennewein's intended use level. Given that 3'-SL levels are reported to be relatively stable throughout lactation, there may be an additional justification for Jennewein's intended use level based on the published literature. Please provide any additional rationale, including any specific published data and data analysis to support the intended higher use level of 3'-SL.*

   To understand the relationship between the intended use level in GRN 000921 of 0.28 g/L, the intended use levels in GRN 000776 and GRN 000880 of 0.238 and 0.2 g/L, respectively, and the amount of 3'-SL that has been reported in the publicly available literature, the mean, median, central 68% confidence interval, 90th percentile, and distribution of the reported means or medians were calculated in a non-parametric analysis using the following:

   1. Means or medians reported in the studies included in Table 7;
   2. Means reported in the studies included in the systematic review conducted by Thurl et al. (2017);
   3. Means or medians reported in other publicly available studies (Austin et al., 2019; Alderete et al., 2015; Larsson et al., 2019; McJarrow et al., 2019; Paganini et al., 2019; Samuel et al., 2019; Williams et al., 2017).

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4. Means or medians reported in selected studies excluded from the systematic review conducted by Thurl et al. (2017) (Austin et al. (2016), Kunz et al. (2017), Marx et al. (2014), Bode et al. (2012), and Kuhn et al. (2015)).

The selected studies that were excluded from the Thurl et al. (2017) systematic review were included in the analysis because 3'-SL levels in breast milk are not affected by secretor status. Additionally, if only medians were reported in a study, then the medians were used in the analysis.

A total of one hundred and thirty-four means and medians were extracted from the included studies and the resulting mean, median, upper and lower limits of the central 68% confidence interval, and 90th percentile were calculated to be 0.23, 0.19, 0.11, 0.31, 0.38 g/L, respectively. The distribution of the means is presented in Figure 1. The intended use level of 0.28 g/L falls within one standard deviation above the reported means/medians and therefore mimics a greater percentage of the breast milk 3'-SL levels in the population than the intended use levels of 0.23 and 0.2 g/L specified in GRN 000776 and GRN 000880. It is important to note that all the studies included in the analysis used different analytical methods to quantify the levels of 3'-SL. Thus, although there is variability in the 3'-SL levels in the breast milk obtained from each participant in each study, there may be additional variability across the different studies due to the use of different analytical methods.

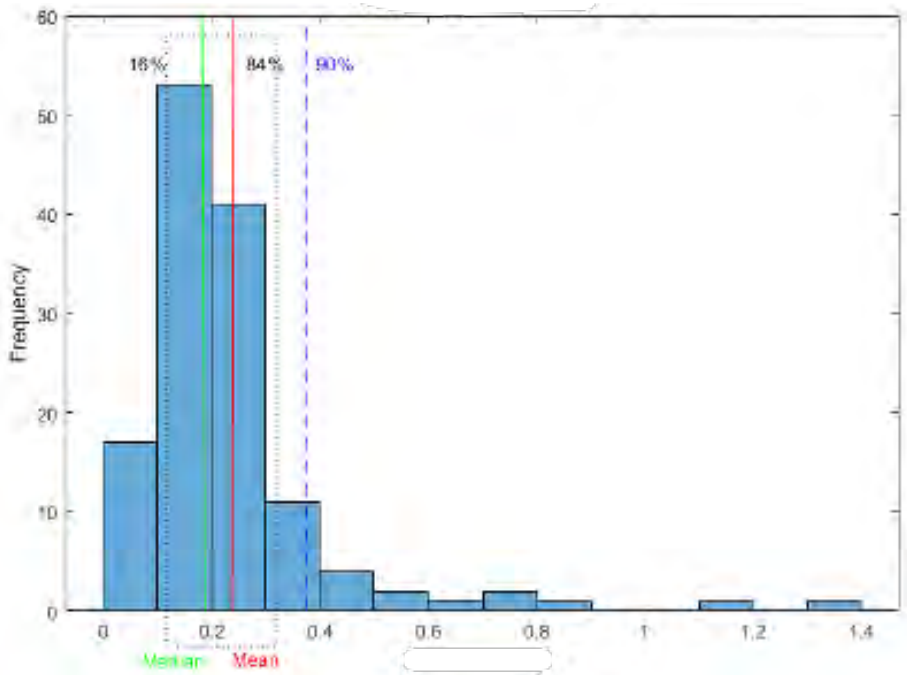


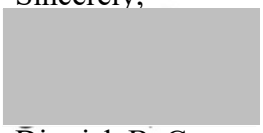
Figure 1. *The distribution, mean, median, the central 68% confidence interval, and 90th percentile of the mean/median levels of 3'-SL in human milk. One hundred and thirty-four means and medians were abstracted from the studies included in Table 7 of GRN 000921, reviewed by Thurl et al. (2017), as well as other publicly available studies. The histogram depicts the distribution of the 3'-SL means/medians and the corresponding location of the mean (red line), median (green line), the upper and lower limits of the central 68% confidence interval (box with black dotted line), and the 90th percentile (blue dashed line).*

2. *In the response to our questions, Jennewein states that E. coli BL21(DE3) strain "JBT-3SL" is deposited in the "DMSZ – German Collection of Microorganisms and Cell Cultures GmbH". Please clarify if the deposit designation is supposed to be "DSMZ – German Collection of Microorganisms and Cell Cultures GmbH"?*

   DMSZ - German Collection of Microorganisms and Cell Cultures GmbH was a typographical error. The deposit designation should be "DSMZ – German Collection of Microorganisms and Cell Cultures GmbH".

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

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