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 6'-Sialyllactose Sodium Salt (6'-SL) in Non-Exempt Term Infant Formula constitutes a new notification. Although 6'-SL is the subject of other GRAS Notices, the subject of the enclosed GRAS Notice is produced using a novel production process.

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 6'-Sialyllactose Sodium Salt (6'-SL) in Non-Exempt Term Infant Form, and all references

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GRAS Determination for the Use of 6'-Sialyllactose Sodium Salt in Non-Exempt Term Infant Formula

Prepared for:

Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

Prepared by:

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

March 19, 2020

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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*-*neo*hexaose

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

6'-Sialyllactose Sodium Salt (6'-SL)

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

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

E. INTENDED USE

Jennewein Biotechnologie intends to use 6'-SL as a substitute for other forms of 6'-SL in cow's milk-based, non-exempt infant formula for term infants at a level of 0.4 g/L.

F. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of 6'-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 6'-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 6'-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 90% 6'-SL dry weight.

    a. 6'-Sialyllactose is a naturally occurring acidic oligosaccharide in human milk.

    b. The 6'-SL that is the subject of this GRAS Notice is structurally identical to the 6'-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 when stored from the date of production under ambient conditions.

2. Human milk oligosaccharides, including 6'-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 6'-SL in breast milk ranges from 0.1 to 0.8 g/L with means and medians ranging from 0.08 to 0.65 g/L and 0 to 1.1 g/L, respectively.

4. Genotoxicology and subchronic toxicology studies published by Phipps et al. (2019) show that 6'-SL is not genotoxic and has a no observed adverse effect level (NOAEL) of 5 g/kg bw/day, which was the highest dose tested.

5. The addition of 0.4 g/L 6'-SL in infant formula will result in mean and 90th percentile estimated daily intakes (EDI) of 0.49 g/day (70.5 mg/kg bw/day) and 1.02 g/day (143.0 mg/kg bw/day) for 0 to 6 month-old infants, which represents the maximum level of intake because infants at this age consume infant formula as a sole source of nutrition.

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

    a. Published studies that quantitate the levels of 6'-SL in human milk;

    b. Analytical data demonstrating that the 6'-SL produced by Jennewein is structurally identical to 6'-SL from human milk;

    c. Data demonstrating the qualitative and quantitative similarities between the subject of this GRAS Notice and the 6'-SL ingredient tested by Phipps et al. (2018), which is the subject of GRN 881;

    d. Corroborative published genotoxicology and 90-day subchronic toxicology studies conducted with 6'-SL or a mixture of human milk oligosaccharides containing 4.0 % of Jennewein-manufactured 6'-SL.

    e. A corroborative unpublished tolerance study in neonatal piglets conducted with a mixture of HMOs containing up to 0.34 g/L of Jennewein's 6'-SL ingredient that showed an HMO mixture containing 6'-SL was well-tolerated and supported normal growth in neonatal piglets.

Therefore, 6'-SL is safe and GRAS at the proposed level of addition to the intended non-exempt, term infant formula. 6'-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

6'-Sialyllactose sodium salt (6'-SL; CAS No. 35890-39-2)

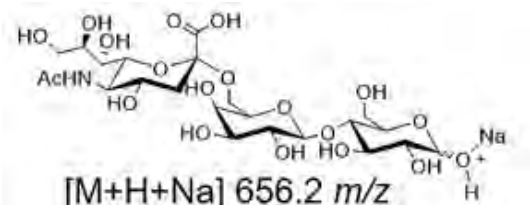
B. CHEMICAL NAME

N-acetyl α-neuraminic-(2→6)-β-D-galactose-(1→4)-D-glucose sodium salt

C. MOLECULAR FORMULA AND MASS

$C_{23}H_{39}NNaO_{19;}$ 633.55 g/mol

D. STRUCTURAL FORMULA



E. DESCRIPTION OF 6'-SL

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, 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 non-reducing terminus via α2,3 or α2,6 bonding, respectively.

The subject of this notice is a 6'-SL sodium salt produced by fermentation using a genetically engineered strain of *Escherichia coli* BL21 (DE3). The 6'-SL sodium salt is then purified from the culture medium and spray-dried, producing a powdered finished product. The finished product contains not less than 90% 6'-SL and the structure of 6'-SL present in the finished product is consistent with 6'-SL as confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, double-quantum filtered $^{1}H^{1}H$-COSY NMR spectroscopy, phase-sensitive $^{1}H^{13}C$-heteronuclear single quantum correlation (HSQC) NMR spectroscopy,

phase-sensitive $^{1}H^{13}C$-heteronuclear multiple bond correlation (HMBC) NMR spectroscopy, and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). Residual impurities include lactose and carbohydrate by-products.

Additionally, a 6'-SL sodium salt is GRAS and the subject of GRAS Notification (GRN) 881. As of March 20, 2020, FDA's letter for GRN 881 is pending.

F. PRODUCTION PROCESS

The subject of this GRAS Notification is produced by fermentation using *JBT-6SL* a genetically engineered strain of *E. coli* BL21 (DE3). The 6'-SL sodium salt is then purified from the fermentation medium and the final product is spray-dried.

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-6SL*. All genes integrated into the Basic strain are well-characterized. Additionally, all plasmids and/or episomal vectors were removed during the engineering process. The strain is stored at the production site as glycerol stock in a master cell bank at -80°C. The strain will be deposited at the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen)-German Collection of Microorganisms and Cell Cultures. The glycerol stocks are used to produce working cell banks, which are then used to produce the finished ingredient.

a. The Basic Strain

To generate the Basic strain, endogenous genes encoding a β-galactosidase, L arabinose-isomerase, L-fucose isomerase, L-fuculokinase, *N*-acetylglucosamine 6-phosphate deacetylase, glucosamine 6 phosphate deaminase, lipopolysaccharide biosynthesis protein, and UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase were either inactivated by mutagenesis using mismatched oligonucleotides or deleted by homologous recombination (Table 1). In contrast, genes encoding a UDP-galactose-4-epimerase, galactosyltransferase, galactokinase, galactose mutarotase, and lactose permease were amplified from *E. coli* K12 genomic DNA and integrated by either site-specific homologous recombination or transposition (Table 1) (Datsenko and Wanner, 2000; Lampe et al., 1999). Arabinose isomerase (*ara*A) was also inactivated by mutagenesis using mismatch oligonucleotides to prevent L-arabinose degradation (Ellis et al., 2001) and allow for arabinose-induced expression of λ red recombinase and transposase required for transposition. The antibiotic resistance genes that were integrated during homologous recombination and 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 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 the Basic Strain

Gene Product Name	Origin of the Gene	Manipulation	Effect
β-galactosidase	*E. coli* BL21(DE3)	Deletion	To prevent hydrolysis of lactose
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. Generation of JBT-6SL

In the production strain *JBT-6SL*, 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 glutamine fructose-6-phosphate aminotransferase from *E. coli* K12, glucosamine-6-phosphate *N*-acetyltransferase from *Saccharomyces cerevisiae*, *N*-acetylglucosamine 2-epimerase from *Synechocystis* sp. PCC6803, *N*-acetylneuraminic acid synthase from *C. jejuni*, CMP-N-acetylneuraminic acid synthase from *Campylobacter jejuni*, and 2,6-sialylltransferase from *Streptococcus suis* were introduced by transposition. 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 to nitrosoguanidine (NTG) mutagenesis and screened for their ability to produce high levels of 6'-SL, resulting in *JBT-6SL*. 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 growth at 42°C, ampicillin sensitivity, and failure to amplify plasmid specific DNA. All integrated genes remain in the genome and, although *JBT-6SL* possesses the dihydrofolate reductase, bleomycin resistance, neomycin-phosphotransferase II, and gentamycin 3'-acetyltransferase genes used for integrant selection, no plasmids or episomal vectors remain in the genome.

Table 2. Genetic Manipulations in *JBT-6SL*

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	
α2,6-sialylltransferase	*Streptococcus suis*	Ectopic expression	Confer 6'-sialyllactose 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 6'-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, as well as other GMP-, ISO-, and International Featured Standards Food 6.1-compliant manufacturers, which have been audited and approved by Jennewein. Jennewein is also a Food Facility registered with the FDA (Registration # 1303109037512).

b. Processing Aids and Food Contact Surfaces

All raw materials, processing aids, and food contact substances used to produce 6'-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 the 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 some process parameters, 6'-SL is manufacturing using the same process as the 2'-FL 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 (see Section 2.2.2 of GRN 571, pg 5 – 9). Briefly, production of 6'-SL involves three steps (Figure 1). During Step 1, *JBT-6SL* is expanded in minimal medium containing a carbon source consisting of glucose, sucrose, glycerol, or a combination thereof, and then during fermentation, lactose is added preferentially in 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 6'-SL concentrate into powder.

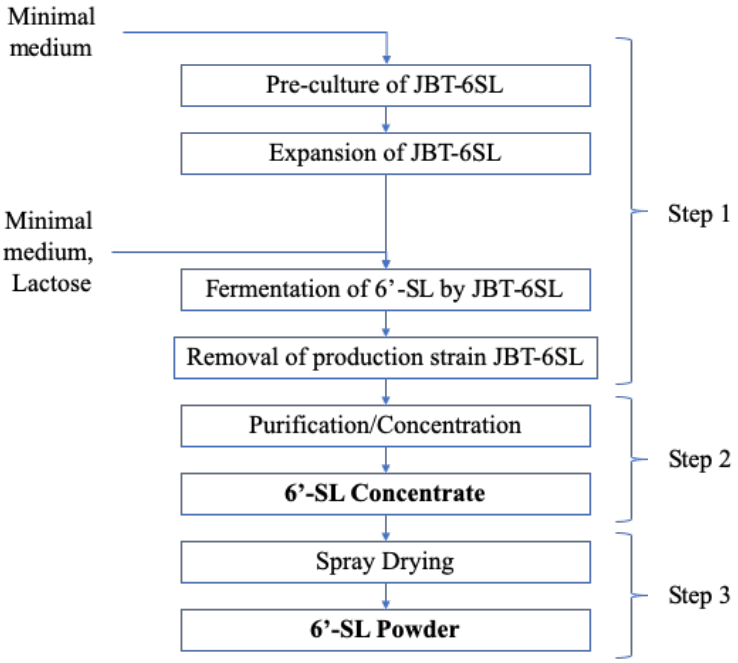


Figure 1. Production Process for 6'-Sialyllactose

A genetically modified strain of *E. coli* BL21(DE3), *JBT-6SL*, is expanded in minimal medium with the addition of lactose to the culture, 6'-SL is produced. The production strain/biomass is then removed yielding the 6'-SL-containing fermentation medium. The medium is then purified and concentrated in a series of filtration, ion exchange, electrodialysis, and decolorization steps yielding the 6'-SL concentrate. The concentrate is then spray dried, producing a powder containing 6'-SL.

G. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. 6'-SL Product Specifications and Batch Data Compliance

To ensure a consistent genetically modified organism-free food-grade product, each batch of 6'-SL is evaluated against a set of product specifications, which control the amount of 6'-SL, carbohydrate by-products, DNA and endotoxin residues derived from the production strain, heavy metals, and microbes (Table 3). Each parameter is measured using either compendial or 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 five non-consecutive batches of 6'-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 for 6'-Sialyllactose

Parameter	Method	Specification					
			11020039	11020049	11020059	11020069	
Appearance (Color)[4]	Visual	White to ivory Colored	Complies	Complies	Complies	Complies	Complies
Appearance (Form)[4]		spray-dried powder	Complies	Complies	Complies	Complies	Complies
6'-Sialyllactose[4]	HPAEC-PAD	≥ 90% DW	94.6	94.3	92.3	95.4	95.8
Sum of other carbohydrates[4]		≤ 10%	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
D-lactose[4]		≤ 5%	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sialic acid[4]		≤ 10%	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
N-Acetylglucosamine[4]		≤ 5%	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Protein[4]	Nanoquant (modified Bradford)	≤ 100 µg/g	16.8	16.8	14.4	16.6	16.0
Ash[1]	ASU L 06.00-4	≤ 8.5%	5.7	3.8	6.4	6.6	5.7
Moisture[4]	KF titration	≤ 9.0%	7.7	7.8	7.6	8.0	8.2
Sodium[1]	PV-347 ICP-MS	≤ 4.2 %	3.0	3.1	3.5	3.1	3.2
Endotoxins[3]	Ph. Eur. 2.6.14	≤ 10 EU/mg	0.034	0.014	0.034	0.016	0.009
Aflatoxin M1[1]	DIN EN ISO 14501	≤ 0.25 µg/kg	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
GMO residues[2]	qPCR	Negative	Negative	Negative	Negative	Negative	Negative
Arsenic[1]	ASU L 00.00-135 – ICP-MS	≤ 0.2 mg/kg	ND	ND	ND	ND	ND
Cadmium[1]		≤ 0.1 mg/kg	ND	ND	ND	ND	ND
Lead[1]		≤ 0.02 mg/kg	ND	ND	ND	ND	ND
Mercury[1]		≤ 0.5 mg/kg	ND	ND	ND	ND	ND
Standard Plate Count[1]	ISO 4833-2	≤ 10000 cfu/g	< 10	< 10	< 10	< 10	< 10
Yeast and Molds[1]	ISO 21527-2	≤ 100 cfu/g	< 20	< 20	< 20	< 20	< 20
Coliform/*Enterobacteriaceae*[1]	ISO 21528-1	≤ 10 cfu/g	< 10	< 10	< 10	< 10	< 10
Salmonella[1]	ISO 6579	Negative/25 g	Absent	Absent	Absent	Absent	Absent
Cronobacter sakazakii spp.[1]	ISO/TS 22964	Negative/10 g	Absent	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 analysis

Although the oligosaccharide-containing media is subjected to ion exchange chromatography and electrodialysis to minimize the amount of elements in the finished product, Jennewein analyzed five batches of the finished 6'-SL product for the levels of manganese, selenium, iron, copper, molybdenum, nickel, zinc, and cobalt (Table 4). Manganese, selenium, iron, molybdenum and cobalt, which are all media components, were all below the limit of detection. 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 6'-Sialyllactose

Element[1]	Method	Batch Number				
		11021019	11020039	11020049	11020059	11020069
Manganese (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 1.7	< 1.7	< 1.7	< 1.7	< 1.7
Selenium (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Iron (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 0.61	< 0.61	< 0.61	< 0.61	0.96
Copper (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 0.59	1.4	1.6	< 0.59	1.2
Molybdenum (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 0.06	< 0.06	< 0.06	< 0.06	< 0.06
Nickel (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.086	0.08	0.095	0.12	0.086
Zinc (mg/kg)	ASU L 00.00-135 (ICP-MS)	3.2	1.5	12.3	9.1	5.2
Cobalt (mg/kg)	PV-347 ICP-MS	< 0.04	< 0.04	< 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; copper LOQ = 0.59 mg/kg; iron LOQ = 0.6 mg/kg; molybdenum LOQ = 0.06 mg/kg; cobalt LOQ = 0.04 mg/kg.

H. STABILITY OF 6'-SIALYLLACTOSE

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-6SL* 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 6'-SL

The shelf-life of 6'-SL is supported by ambient (25°C and 60% relative humidity) and accelerated (40°C and 75% relative humidity) stability studies conducted with the 6'-SL that is the subject of GRN 881. Ambient (25°C and 60% relative humidity) and accelerated (40°C and 75% relative humidity) stability studies have also been conducted on a mixture of HMOs containing Jennewein's 6'-SL. Because Jennewein's 6'-SL is compositionally similar to the subject of GRN 881, it is reasonable to expect that the stability of Jennewein's will be similar to the 6'-SL that is the subject of GRN 881. Therefore, the ambient and accelerated stability studies presented in GRN 881 are incorporated by reference and briefly summarized below, along with the ambient and accelerated stability studies conducted on the 6'-SL containing mixture of HMOs.

In the ambient stability studies conducted on the 6'-SL that is the subject of GRN 881, chemical, physical, microbiological, and sensory testing was conducted on 2 representative batches of the finished ingredient over the course of 12 months (see pg. 18 of GRN 881). For both batches a complete set of chemical (moisture, 6'-SL, lactose, sialic acid, 6'-sialyl-lactulose, and unspecified impurities), physical (color and appearance), and microbiological (aerobic mesophilic total plate count, Enterobacteriaceae, *Salmonella*, *Cronobacter* (*Enterobacter) sakazakii*, *Listeria monocytogenes*, *Bacillus cereus*, Yeasts, and Molds) data was available at 12 months. Additional data was provided for the chemical and physical parameters at months 0, 3, 6, and 9. All parameters tested complied with the product specifications. Thus, it is reasonable to conclude that ingredient will be stabile when stored under ambient conditions for at least 12 months.

In the accelerated stability studies conducted on the 6'-SL that is the subject of GRN 881, chemical, physical, microbiological, and sensory testing was conducted on two representative batches of finished ingredient over the course of 12 months (see pg. 21 of GRN 881). The results show that there are no changes in the organoleptic properties of 6'-SL, no appreciable degradation of 6'-SL, no changes in the impurity profile, and no alterations in the microbiological quality of the ingredient following storage over the course of 12 months under defined, accelerated storage conditions.

To understand the stability of Jennewein's 6'-SL when combined with other HMOs, a mixture containing 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), 3'-sialyllactose (3'-SL), lacto-*N*-tetraose (LNT), and 4% 6'-SL 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. 6'-Sialyllactose and moisture content were monitored overtime using the same methods that are used for batch qualification.

Although there was analytical variability, 6'-SL 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, 6'-SL decreased, and moisture increased over the course of the 26-week testing period (Table 6).

Additionally, because the stability studies summarized in GRN 766 indicate that the 3'-SL, another acidic oligosaccharide, is also stable for at least 1 year under ambient conditions and at least 3 months under accelerated conditions (GRN 766), significant changes in the stability of the 6'-SL that is the subject of this GRAS notification are not expected. Thus, these results together support a shelf-life for 6'-SL of 1 year from the date of production when stored under ambient conditions.

Table 5. Stability of 6'-Sialyllactose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Ambient Conditions (25°C, 60% Relative Humidity)

Batch 4011-1004303107		Moisture content		6'-SL	
		%	% of baseline	% DW	% of baseline
Interval	Baseline	5.7	100.0	5.63	100
	Week 1	5.2	91.9	5.22	92.7
	Week 4	6.2	109.2	5.54	98.3
	Week 8	6.1	108.3	5.10	90.6
	Week 13	6.1	107.2	6.22	110.5
	Week 26	6.9	121.7	4.93	87.5
	Week 39	7.3	129.3	5.88	104.5
	Week 52	7.8	137.0	5.88	97.6

Abbreviations: DW, dry weight; 6'-SL, 6'-sialylactose; NA, not applicable.

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

Batch 4011-1004303107		Moisture content		6'-SL	
		% DW	% of baseline	% DW	% of baseline
Interval	Baseline	5.7	100.0	5.63	100
	Week 1	5.8	101.4	515	91.5
	Week 4	6.6	117.1	5.42	96.3
	Week 8	7.3	129.1	5.05	89.6
	Week 13	8.7	153.6	6.15	109.2
	Week 26	9.9	174.6	4.68	83.1

Abbreviations: DW, dry weight; 6'-SL, 6'-sialylactose; NA, not applicable.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

The intended effect of adding 6'-SL to term, cow's milk-based, non-exempt infant formula to increase 6'-SL intake in formula-fed infants and promote the growth of beneficial bacteria, including, but not limited to, *Bifidobacteria*.

B. HISTORY OF EXPOSURE

6'-SL is a naturally occurring acidic oligosaccharide found in human milk and is also present at comparable levels in mature bovine, goat, and, to a lesser extent, donkey milk (Martin-Sosa et al., 2003; Claps et al., 2014; Licitra et al., 2019). Thus, humans have been exposed to 6'-SL either through the ingestion of milk from humans or other mammals.

The concentration of 6'-SL in human breast milk has been analyzed in 27 studies (Asakuma et al., 2007; Austin et al., 2016; Austin et al., 2019; 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., 1999; Kunz et al., 2017; Larsson et al., 2019; Leo et al., 2010; McGuire et al., 2017; McJarrow et al., 2019; Martin-Sosa et al., 2003; Nijman et al., 2018; Paganini et al., 2019; Samuel et al., 2019; Smilowitz et al., 2013; Spevacek et al., 2015; Sprenger et al., 2017; Sumiyoshi et al., 2003; Tonon et al., 2019; Thurl et al., 2010; Thurl et al., 2017; Van Niekirk et al., 2004; Wejryd et al., 2018). The results of 14 of these studies were summarized in a recent systematic review conducted by Thurl et al. (2017). A summary of the findings reported by Thurl et al. (2017) and the thirteen additional studies is presented in Table 7.

In the available studies, the average concentration of 6'-SL ranged from 0.1 - 0.8 g/L. Unlike other HMOs, there is no relationship between secretor status and 6'-SL levels, although there is a large variation in reported values due to differences in time postpartum, geographical location or study population. Most longitudinal studies have demonstrated that 6'-SL concentration decreases with lactation time (2- to 10- fold, depending on the study), although this relationship was not observed in Kunz et al. (2017).

Table 7. Studies Determining the Concentration of 6'-Sialyllactose in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	6'-SL concentration
Austin et al., 2016	China	Total 450 donors (~90 donors/timepoint)	Days 5-8, 12-30. Months 1-2, 2-4, 4-8	Reported Range: 0.011-0.69 g/L Highest mean: 0.34 ± 0.14 g/L (Days 5-8) Highest median: 0.34 g/L (Days 5-8) Lowest mean: 0.039 ± 0.21 g/L (Months 4-8) Lowest median: 0.035 g/L (Months 4-8)
Austin et al., 2019	Switzerland	• 27 donors with preterm infants, 280 samples • 34 donors with term infants, 220 samples	Weekly for 8 weeks after delivery (preterm and term) then every 2 weeks until 16 weeks (preterm only)	Preterm • Reported Range: 0.046-1.14 g/L • Highest mean: 0.51 ± 0.28 g/L (postpartum week 2) • Highest median: 0.56 g/L (postpartum week 2) • Lowest mean: 0.10 ± 0.045 g/L (postpartum week 16) • Lowest median: 0.098 g/L (postpartum week 16) Term • Reported Range: 0.025-1.08 g/L • Highest mean: 0.65 ± 0.19 g/L (postpartum week 2) • Highest median: 0.61 g/L (postpartum week 2) • Lowest mean: 0.22 ± 0.13 g/L (postpartum week 8) • Lowest median: 0.19 g/L (postpartum week 8)
Azad et al., 2018	Canada	427 mothers	3-4 months postpartum	Reported Range: 0.02-1.71 g/L Mean: 0.16 ± 0.13 g/L Median: 0.13 g/L
Kunz et al., 2017	Spain	33 donors, each giving milk at three stages of maturity	Colostrum: 1-7 days; Transitional milk: 8-15 days; Mature milk: >16 days	Reported Range: 0.41-0.88 g/L Highest median: 0.67 g/L (Transitional milk) Lowest median: 0.56 g/L (Colostrum and Mature milk)
Larsson et al., 2019	Denmark	Mothers of high weight gain and normal weight gain infants	5-6.5 and 9 months postpartum	Highest median: 0.00016 g/L Lowest median: 0.000013 g/L
McGuire et al., 2017	Ghana, Kenya, Peru, Spain, Sweden, rural and urban Ethiopia and Gambia, Washington State (USA), and California (USA)	410 healthy women	2 weeks to 5 months postpartum	Highest mean: 0.56 ± 0.06 g/L, (Ghana) Lowest mean: 0.13± 0.02 g/L (Sweden)

Table 7. Studies Determining the Concentration of 6'-Sialyllactose in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	6'-SL concentration
McJarrow et al., 2019	United Arab Emirates (UAE)	Healthy women recruited during 3rd trimester of pregnancy, ~40 samples/timepoint	Transitional milk: 5-15 days postpartum; Mature milk: 6 months postpartum	Highest mean: 0.64 ± 0.2 g/L (Transitional milk) Lowest mean: 0.08 ± 0.05 g/L (Mature milk)
Nijman et al., 2018	California, USA	10 healthy women who delivered term infants provided samples on days 3 and 42 postpartum	Days 3, 42 postpartum	Highest mean: 0.34 ± 0.03 g/L (Day 3) Lowest mean: 0.25 ± 0.02 g/L (Day 42)
Paganini et al., 2019	Kenya	• 75 breast milk samples from one time point. • 16 breast milk samples from two timepoints, 3 months apart. Data not separated by secretor status	• Median lactation ages for secretors: 13.8 month postpartum, non secretors: 16.1 months postpartum. • Median lactation ages for first timepoint was 7.9 months and the second timepoint was 10.9 months.	Highest median: 1.1 g/L (Interquartile range 0.8 – 1.1) (first timepoint) Lowest median: 0.0 g/L (Interquartile range 0.0 – 0.0) (second timepoint)
Samuel et al., 2019	Spain, France, Italy, Norway, Portugal, Romania, Sweden	226 mothers	2, 17, 30, 90, and 120 Days postpartum	Reported range: 0.022-1.31 g/L Highest mean: 0.65 ± 0.19 g/L (17 days) Highest median: 0.58 g/L (17 days) Lowest mean: 0.10 ± 0.07 g/L (120 days) Lowest median: 0.09 g/L (120 days)
Sprenger et al., 2017	Singapore	48 mother mothers with term infants	30, 60, and 120 days postpartum	Reported range: 0.04-1.0 g/L Highest mean: 0.56 ± 0.2 g/L (30 days) Highest median: 0.58 g/L (30 days) Lowest mean: 0.12 ± 0.05 g/L (120 days) Lowest median: 0.11 g/L (120 days)

Table 7. Studies Determining the Concentration of 6'-Sialyllactose in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	6'-SL concentration
Thurl et al., 2017	Worldwide	Systematic review including 21 previous studies (not all reported 6'-SL) Total of 122 donors, 365 samples	Lactation days 0 to >100	Term • Highest Mean: 0.47 g/L (0.36 – 0.58 g/L 95% confidence level) • Lowest Mean: 0.14 g/L (0.01 – 0.26 g/L 95% confidence level) Pre-term Mean: 0.6 g/L (0.4 – 0.8 g/L 95% confidence level)
Tonon et al., 2019	Brazil	78 single human milk samples	Days 17-76 postpartum, (median = 32 days)	Highest mean: 0.41 ± 0.15 g/L Lowest mean: 0.37 ± 0.15 g/L
Wejryd et al., 2018	Sweden	91 mothers	• 14 and 28 days postpartum • Postmenstrual week 36	Highest median:0.76 g/L(Day 14) Lowest median: 0.18 g/L (36^{th} PMW)

Abbreviations: 6'-SL: 6'-sialyllactose; Se: secretor; Le: Lewis blood group

C. INTENDED USE

Jennewein intends to use 6'-SL powder in cow's milk-based term, non-exempt infant formula as a source of human milk oligosaccharides.

D. ESTIMATED DAILY INTAKE

Because Jennewein intends to use its 6'-SL powder as a substitute for what is currently marketed in the United States at a level of 0.4 g/L, the same concentration as what is GRAS (GRN 881), we incorporate by reference the estimated daily intakes from GRN 881 (pg 33). Briefly, the addition of 0.4 g 6'-SL powder/L in infant formula will result in mean and 90th percentile estimated daily intakes (EDI) of 0.49 g/day (70.5 mg/kg bw/day) and 1.02 g/day (143.0 mg/kg bw/day) for 0 to 6 month-old infants, which represents the maximum level of intake because infants at this age consume infant formula as a sole source of nutrition.

IV. SELF-LIMITING LEVELS OF USE

This part does not apply.

V. COMMON USE IN FOOD BEFORE 1958

This part does not apply.

VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The general recognition of safety of 6'-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 6'-SL in human milk (see Section III.B); the analytical data demonstrating that the 6'-SL produced by Jennewein is structurally identical to 6'-SL from human milk; the published toxicology studies with Glycom's 6'-SL ingredient, which support the GRAS status of the subject of GRN 881; the qualitative and quantitative similarities between the subject of this GRAS Notice and Glycom A/S's 6'-SL ingredient; the corroborating toxicology studies conducted with HMO mixtures containing Jennewein-manufactured 6'-SL; and other corroborating neonatal piglet and clinical studies that evaluated the tolerability of 6'-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 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 to an important role in promoting the growth of the infant gastrointestinal tract microbiota and maturation of the intestinal mucosal immune system (Kunz et al., 1999; Jost et al., 2015). Structurally, they contain glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), fucose (Fuc), and *N*-acetyl-neuraminic acid moieties (Neu5Ac) (Milani et al., 2017). All HMOs have lactose (Galβ1-4Glc) at the reducing end and, except for trisaccharides, elongated oligosaccharide chains composed of either lacto-*N*-biose (Galβ1-3GlcNAc) or *N*-acetyllactosamine (Galβ1-4GlcNAc) disaccharide units linked by β1-3 or β1-6 glycosidic bonds at the non-reducing end (reviewed in Bode et al., 2012). A β1-6 glycosidic bond between two disaccharide units introduces chain branching. Additionally, lactose and the elongated oligosaccharide chains can be fucosylated via α1-2, α1-3, or α1-4 linkages or sialyllated via α2-3, or α2-6 linkages. Currently, more than 200 different HMOs have been identified and the highest levels of HMOs are found in colostrum (20-25 g/L).

6'-SL is a naturally occurring oligosaccharide in human milk. Numerous published studies have examined the level of 6'-SL in human milk, and the reported range is 0.1 to 0.8 g/L with means and medians ranging from 0.08 to 0.65 g/L and 0 to 1.1 g/L, respectively. Higher levels of 6'-SL are reported during early lactation than in later lactation time. Thus, Jennewein's intended use of 0.4 g 6'-SL/L infant formula is well within the established range of 6'-SL that occurs naturally in breast milk.

Because human milk is the reference standard for infant nutrition, infant formula manufacturers look to mimic the composition and functionality of human milk in their formulas as much as possible. Manufacturing HMOs on a commercial scale, however, has not been feasible until recently and 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 or innate components of breast milk.

Additionally, the use of selected HMOs opposed to a mixture of the almost 200 HMOs in infant formula has been called into question (Milani et al., 2017). However, it is important to note that breast milk is considered to be the reference standard for infant nutrition, both the types and amounts of HMOs in breast milk can vary greatly from one mother to another, and observational studies that investigated the effects of varying breast milk HMO composition on infant growth and health have reported conflicting results due to design limitations and/or confounding factors (Alderete et al., 2015; Azad et al., 2018; Berger et al., 2020; Lagström et al., 2020; Gridneva et al., 2019; Kuntz et al., 2019; Larsson et al., 2019; Sprenger et al., 2017; Vandenplas et al., 2018). Thus, a clear and consistent link between the use of selected and structurally different HMOs in infant formula and adverse outcomes on infant growth and health does not exist. Therefore, based on the totality of the available evidence, it is reasonable to expect that supplementing infant formula with a synthetic form of 6'-SL will not pose risks to infants consuming formula containing 6'-SL.

The safety of 6'-SL as an ingredient in infant formula is supported by a battery of published and unpublished genotoxicity, subchronic toxicity, and tolerability studies on 6'-SL alone or 6'-SL in combination with a variety of other HMOs (Gurung et al., 2018; Phipps et al., 2019; Parschat et al., 2020; unpublished neonatal piglet study). Because Jennewein's 6'-SL ingredient is manufactured in a similar manner, and qualitatively similar and quantitatively comparable to the 6'-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 6'-SL product. 6'-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 subchronic toxicity studies published by Gurung et al. (2018) and Parschat et al. (2020), which were conducted with a 6'-SL product manufactured by GeneChem and a mixture of HMOs containing 6'-SL manufactured by Jennewein, respectively, corroborate the results reported by Phipps et al. (2019), as well as neonatal piglet study conducted with a mixture of HMOs containing 6'-SL manufactured by Jennewein.

Based on these data, there is reasonable certainty that the use of Jennewein's 6'-SL per the intended use and use level is of no harm to consumers. Jennewein's 6'-SL is therefore GRAS as an ingredient in non-exempt, term 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 have been extensively summarized in previous GRAS Notices and opinions published by authoritative bodies around the world (GRN 48, 546, 547, 571, 650, 659,735, 749, 749, 766, 833; EFSA Panel on Dietetic Products, 2015, EFSA Journal, 13, 4183; EFSA Panel on Nutrition et al., 2019, EFSA Journal, 17). Briefly, HMOs 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 and Kunz, 2012; Rudloff et al., 2006; Rudloff et al., 2012; Rudloff et al., 1996; Chaturvedi et al., 2001; Goehring et al., 2014; Ruhaak et al., 2014; Gnoth et al., 2000; Engfer et al., 2000; Brand-Miller et al., 1998; De Leoz et al., 2013). 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 model suggest that neutral HMO are transported across the intestinal epithelium by receptor-mediated transcytosis as well as by paracellular transport, whereas acidic HMO are absorbed via the non-specific paracellular transport only (Gnoth et al., 2001).

Importantly, because the 6'-SL that is the subject of this GRAS Notification is structurally identical to the 6'-SL found in breast milk and the resulting estimated daily intake of 6'-SL from the intended uses approximates 6'-SL intake from breast milk (see Section III.B), there is reasonable certainty that the absorption, distribution, metabolism, and excretion of 6'-SL ingested from the intended uses at the intended use levels will mimic that from breast milk.

C. TOXICOLOGY

The pivotal toxicology studies that support the use of Jennewein's 6'-SL ingredient in infant formula include a battery of genotoxicity and subchronic toxicity studies that were conducted using a 6'-SL-containing ingredient manufactured by Glycom A/S and published by Phipps et al. (2019). Additional corroborating genotoxicity and subchronic toxicity studies published by Gurung et al. (2018) and Parschat et al. (2020) have been conducted using an 6'-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* mammalian cell micronucleus test, and an OECD-compliant 90-day feeding toxicity study with a product containing 96.8% 6'-SL manufactured by Glycom A/S to support the GRAS status of the subject of GRN 881. Gurung et al. (2018) conducted the corroborating FDA Redbook-compliant bacterial reverse mutation, chromosomal aberration, and *in vivo* micronucleus assays, and an acute oral toxicity and a 90-day dietary toxicity study to support the safety of a product containing >98 % 6'-SL GeneChem Inc. Parschat et al. (2020) evaluated the genotoxicity and subchronic toxicity of Jennewein's 6'-SL in combination with 2'-FL, 3'-FL, LNT, and 3'-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 6'-SL used 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 6'-SL, and had 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 6'-SL. Additionally, 6'-sialyllactitol and 6'-sialyl-lactulose, possible degradation products of Glycom A/S's 6'-SL ingredient that is the subject of GRN 881 and were used by Phipps et al. (2019), are not expected in Jennewein's product due to the 6'-SL production process.

Table 8. Comparison of Jennewein's 6'-Sialyllactose Sodium Salt With the 6'-Sialyllactose Sodium Salt Tested Phipps et al. (2019) and That Supports GRN 881

Parameter	GRN 881		Jennewein's Specifications
	Phipps et al., 2019	Specifications	
6-Sialyllactose	96.8 %	≥ 90.0 %	≥ 90.0%
Sodium	3.23 %	2.5 – 4.5% w/w	≤ 4.2 %
Lactose	2.52 %	≤ 5 %	≤ 5%
Sialic Acid	0.51 %	≤ 2.0 %	≤ 10%
6'-Sialyllactulose	0.75 %	≤ 3 %	NS
N-acetylglucosamine	NP	NS	≤ 5%
6'-Sialyl-lactitol	0.04 %	NS	NS
Others	0.54 %	≤ 3 %	NS
Sum of other carbohydrates	3.12 %[a]	NS	≤ 10%[c]
Protein	NP	≤ 100 µg/g	≤ 100 µg/g
Ash	NP	NS	≤ 8.5%
Moisture	1.04 %	≤ 6.0%	≤ 9.0%

NS: not specified; 6'-SL: 6'-Sialyllactose: NP: not provided.
[a]Includes lactose, sialic acid, 6'sialyl-lactulose, 6'-sialyl-lactitol, and others.
[c]Includes lactose, sialic acid, and *N*-acetylglucosamine.

Because Jennewein's 6'-SL ingredient is qualitatively comparable and quantitatively similar to the 6'-SL product manufactured by Glycom A/S and tested by Phipps et al. (2019), the results published by Phipps et al. are the pivotal results that support the safety of Jennewein's 6'-SL product. Additionally, although they tested a 6'-SL ingredient manufactured by enzymatic synthesis and a mixture containing lower amounts of 6'-SL, the results reported by Gurung et al. (2018) and Parschat et al. (2020) corroborate the findings reported by Phipps et al. Briefly, 6'-SL is not genotoxic, not acutely toxic following a single administration of 5000 mg/kg, and does not result in toxicologically significant or treatment-related effects in growth, feed intake or efficiency, clinical observations, or clinical or anatomic pathology changes at 5 g/kg/day. Brief summaries of the studies and results published by Phipps et al., Gurung et al., and Parschat et al. are provided below.

1. Genotoxicity

a. Studies of 6'-Sialyllactose as a Single Ingredient

i. Bacterial Reverse Mutation Assay

The mutagenic activity of 6'-SL was assessed in two published reports: Gurung et al., (2018) performed the bacterial reverse mutation assay according to FDA Redbook 2000 guidelines and Phipps et al., (2019) conducted the assay in accordance with OECD Guideline No. 471 (OECD, 1997). 6'-SL was not mutagenic under the conditions tested in either study.

Phipps et al. (2019) tested the following levels of 6'-SL using both the plate incorporation method and the pre-incubation method, with and without S9 metabolic activation: 0 (water, vehicle control), 5, 15, 50, 150, 500, 1500, and 5000 µg/plate. The following bacterial strains were used in the assay: *Salmonella enterica* serovar *Typhimurium* TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 *uvrA* pKM101. Sodium azide, 9-aminoacridine, 2-nitrofluorine and 4-nitroquinoline n-oxide were used as positive controls in the absence of s9 and benzo(a)pyrene and 2-aminoanthracene were used in the presence of S9 activation. All conditions were performed in triplicate. No increase in revertant frequencies at any test article dose while positive controls induced increases in mean revertant colony numbers. Growth inhibition was not reported in any strain used in the assay.

Gurung et al. (2018) tested the following levels of 6'-SL using the plate incorporation method, with and without S9 metabolic activation: 0 (normal saline, solvent control), 100, 300, 625, 1250, 2500, and 5000 µg/plate. Five strains of *Salmonella typhimurium* were used in the assay: TA97, TA98, TA100, TA102, and TA1535. 4-Nitro-O-phenylenediamine, daunomycin, sodium azide, and methyl methanesulfonate were used as positive controls in conditions without S9 mix, and 2-aminofluorene, 1,8-dihydroxyanthraquinone, and 2-aminoanthracene as positive controls in conditions with S9 mix. All conditions were performed in triplicate. No increase in revertant colony frequency was observed at any test article dose while positive controls increased revertant colonies. However, 6'-SL inhibited TA98 growth at 2500 and 5000 µg/plate.

ii. Chromosomal Aberration Assay

Gurung et al. (2018) performed a chromosomal aberration assay in Chinese hamster lung (CHL) cells according to the FDA Redbook 2000 guidelines. CHL cells were exposed to 0, 225, 450, and 900 µg/mL 6'-SL with and without S9 metabolic activation in two separate assays. Mitomycin C and cyclophosphamide were used for positive control substances in the absence or presence of S9 activation, respectively. 6'-Sialyllactose did not inhibit cell growth at any dose. No increases in structural or numerical chromosomal aberrations while positive controls increased the numbers of structurally aberrant cells. Therefore, 6'-SL was not genotoxic under the conditions of this assay.

iii. Mammalian Micronucleus Test

The ability of 6'-SL to induce micronuclei was assessed in two published reports: in an *in vivo* micronucleus assay in Kunming mice according to FDA Redbook 2000 guidelines (Gurung et al., 2018) and an *in vitro* micronucleus assay in human peripheral blood lymphocytes in

accordance with OECD Guideline No. 487 (OECD, 2016; Phipps et al., 2019). 6'-Sialyllactose did not induce micronucleus formation under the conditions tested in either study.

An *in vivo* mammalian micronucleus test was performed in 4 to 5 week-old male and female Kunming mice administered 0 (purified water, vehicle control), 500, 1000, 2000 mg/kg/day of 6'-SL for two consecutive days at 18 hour intervals. The positive control was 40 mg/kg cyclophosphamide. No clinical signs of toxicity and mortality were observed at any tested dose levels. No statistically significant changes were observed in mean body weights between test groups. 6'-Sialyllactose did not induce significant changes in percentage micronucleated polychromatic erythrocytes or polychromatic erythrocytes/normochromatic erythrocytes in either male or female mice compared to controls. The mice administered cyclophosphamide, the positive control, had a statistically significant increase in the micronucleated polychromatic erythrocytes compared to the vehicle control group.

An *in vitro* mammalian cell micronucleus test was performed in human peripheral blood lymphocytes from healthy, non-smoking adult donors, +/- S9 activation exposed to 0 (water, vehicle control), 500, 1000 or 2000 µg/mL 6'-SL. Positive controls were also included (mitomycin C and colchicine in the absence of S9; cyclophosphamide in the presence of S9). Duplicate cultures were prepared for each test group, and quadruplicate cultures were prepared for vehicle control groups. No cytotoxicity was observed at any concentration of 6'-SL. No difference in percentage of micronucleated cells between 6'-SL and vehicle controls was observed. Positive controls induced statistically significant increases in the number of binucleate cells containing micronuclei compared to vehicle controls.

b. Studies of Jennewein's 6'-Sialyllactose 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 0.2, 0.4, 1.3, 4, 12.6, and 24 mg 6'-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 mg 6'-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 6'-SL contained therein, was not mutagenic under the conditions tested.

Table 9. Bacterial Reverse Mutation Test Performed with an HMO Mixture Containing 4.0% 6'-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 also contains 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-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 0.3, 0.6, 1.2, and 2.4 mg 6'-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 (2.4 mg/mL 6'-SL).

Table 10. *In vitro* Micronucleus Test in Human Peripheral Blood Lymphocytes Exposed to an HMO Mixture Containing 4.0% 6'-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]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, and 5.1% dry weight other carbohydrates manufactured by Jennewein.

2. Toxicity Studies on 6'-SL as a Single Ingredient

a. Acute Oral Toxicity Study

In a FDA Redbook 2000 compliant acute oral toxicity study, the 6'-SL product manufactured by GeneChem Inc. was administered to Sprague Dawley rats by gavage at a single dose of 0, 5, 10, 15, or 20 g/kg body weight (Gurung et al., 2018). No animals died during the 14-day observation period and no clinical signs of abnormality at any dose level were observed. No obvious abnormal clinical signs (i.e., changes in skin color, eyes, mucous membranes, or behavior patterns; loss of fur, or scabbing) were observed in any of 6′-SL groups. No significant differences in mean body weight, feed consumption and water intake, and organ weights among the four test and control groups were found. After termination, macroscopic examinations showed no treatment-related abnormalities. The LD50 of 6′-SL was above 20 g/kg body weight, the highest dose tested.

b. Subchronic Toxicity Studies

There have been two published 90-day oral toxicity studies: one in adult rats (Gurung et al., 2018) and one in neonatal rats (Phipps et al., 2019). The NOAEL was at least 5 g/kg body weight/day, the highest dose tested in both studies.

i. Subchronic Oral Toxicity Study in Adult Rats

A 90-day oral toxicity study was conducted in 6 – 7 week old Sprague Dawley rats according to the FDA Redbook 2000 guidelines (Gurung et al., 2018). Animals (n = 11 rats/sex/group) received 0 (control, purified water) 1.0, 2.5, or 5.0 g/kg body weight 6'-SL via oral gavage daily for 90 days. No clinical signs of toxicity or mortality related to 6′-SL administration at any dose level were observed. No differences were observed in body weight or feed consumption in the 6′-SL and control groups. Ophthalmoscope examinations and urinalysis did not indicate any treatment-related adverse effects.

Although there were some small, statistically significant differences between treated groups and controls for some clinical chemistry parameters (total protein, urea nitrogen, chloride, creatine, alkaline phosphatase, cholesterol and globulin), the differences were within historical data ranges, were not dose-dependent, and were therefore considered incidental changes/biological variations and not treatment-related adverse effects. Similarly, there were small, statistically significant differences between 6'-SL fed groups and control in some hematology parameters (red blood cell count, platelet count, white blood cell count, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, and mean corpuscular volume), but these differences were not considered treatment-related because they were within the historical data ranges and were not dose-responsive in test substance-treated groups.

Both absolute and relative weights of adrenals in the 2.5 g/kg/day male group were significantly lower than in the control group ($p<0.05$) were observed. Relative weights of spleen were lower in the low-dose female group compared with the control group ($p<0.05$). However, the changes were not considered treatment-related because they did not occur in both sexes, were not dose-responsive, and were within the historical control data range. The no-observed-adverse-effect level (NOAEL) was 5.0 g/kg/day in rats.

ii. Subchronic Oral Toxicity Study Neonatal Rats

A 90-day oral toxicity study in neonatal Sprague Dawley rats was performed by Phipps et al. (2019) in accordance with OECD 408 guidelines, with the exception of the age at which the rats received their first dose (7 days of age to 2 weeks before weaning). Animals (n = 10/sex/group) received daily oral gavage of 6'-SL at doses of 0 (sterile water, vehicle control), 1000, 3000, and 5000 mg/kg/day. An additional 5 animals/sex were allocated to receive the

vehicle control, 5000 mg/kg/day 6'-SL, or 5000 mg/kg/day fructooligosaccharide (FOS) for 90 days before being monitored over the course of a 4 week recovery period.

One male in the FOS group was euthanized on study day 88 after showing adverse clinical signs. One male in the 5000 mg/kg/day group was found dead on study day 20 with no notable microscopic or macroscopic findings. These deaths were not attributed to the administration of FOS or 6'-SL. No other mortalities or adverse clinical signs were noted in any group. There were no significant differences in body weight or feed consumption among 6'-SL and control groups. Time of completion for balano-preputial separation was significantly longer ($p<0.05$) for males in the 1000 mg/kg/day group; time of completion for vaginal opening was significantly shorter ($p<0.01$) for females receiving 3000 mg/kg/day; and body weight at time of vaginal opening was significantly lower ($p<0.05$) for females in all 6′-SL groups compared to control animals. These differences were not considered biologically relevant due to lack of dose response. Pre-weaning development, mean ulna growth, and behavioral observations, including Morris maze performance, were unaffected by administration of 6′-SL.

In males, hemoglobin was significantly decreased ($p<0.05$) in all groups receiving 6'-SL versus control. In females, red blood cell count, hemaglobin, and hematocrit were significantly decreased ($p<0.05$) in animals receiving 5000 mg/kg/day; platelets were significantly decreased ($p<0.05$) in all groups receiving 6'-SL; and eosinophils were significantly increased ($p<0.05$) in animals receiving 5000 mg/kg/day 6'-SL. In males, prothrombin time was significantly decreased ($p<0.01$) in animals receiving 5000 mg/kg 6'-SL. In females, activated partial thromboplastin time was significantly increased in animals receiving 6'-SL. No other significant differences in coagulation parameters were noted, and no differences were attributed to 6'-SL. These differences were attributed to random biological variation and were not present at the end of the recovery period.

In males, potassium was significantly decreased ($p<0.01$) in the 5000 mg/kg/day group; chloride was significantly decreased ($p<0.01$) in the 3000 and 5000 mg/kg/day groups; total bilirubin was significantly decreased ($p<0.05$) in the 1000 and 3000 mg/kg/day groups; aspartate aminotransferase (AST) was significantly increased ($p<0.05$) in all 6'-SL fed groups; total protein was significantly decreased in all groups ($p<0.05$ for 3000 mg/kg/day, $p<0.01$ for 1000 and 5000 mg/kg/day); albumin/globulin (A/G) ratio was significantly increased in the 3000 ($p<0.05$) and 5000 ($p<0.01$) mg/kg/day groups; and total cholesterol was significantly decreased ($p<0.05$) in the 5000 mg/kg/day group versus control. These differences were not attributed to 6'-SL administration or were not considered adverse due to lack of differences following the recovery period. In females, chloride was significantly decreased ($p<0.05$) in the 3000 and 5000 mg/kg/day groups; creatinine was significantly decreased in the 5000 mg/kg/day group; total protein was significantly decreased in all 6'-SL groups ($p<0.05$ for 1000 and 3000 mg/kg/day, $p<0.01$ for 5000 mg/kg/day); and albumin was significantly decreased ($p<0.05$) in all 6'-SL

groups compared to control. These differences were not attributed to 6'-SL administration and were not considered adverse due to lack of differences following the recovery period.

In males, urinary total protein was significantly decreased ($p<0.05$) in the 5000 mg/kg/day group compared to control. In females, urinary pH was significantly increased in all 6'-SL groups ($p<0.05$ for 1000 and 3000 mg/kg/day, $p<0.01$ for 5000 mg/kg/day) and urinary total protein was significantly decreased ($p<0.05$) in the 5000 mg/kg/day compared to control. No other significant differences in urinalysis parameters were observed and there were no differences at the end of the recovery period.

In males, the relative (to BW) weight of the liver was significantly increased ($p<0.01$) in the 3000 and 5000 mg/kg/day groups. In females, the relative weight of the heart was significantly decreased ($p<0.05$) in the 3000 and 5000 mg/kg/day groups. The relative weight of the salivary glands was also significantly decreased in the 3000 ($p<0.05$) and 5000 ($p<0.01$) mg/kg/day groups versus control. These differences were not considered related to 6'-SL administration. The NOAEL for 6'-SL was established as 5000 mg/kg bw, the highest dose tested.

3. Toxicity Studies on Jennewein's 6'-SL as Part of an HMO Mixture

a. Seven-day Dietary Toxicity Study

In a seven-day pilot feeding toxicity study, female CD/Crl:CD rats (Charles River Laboratories, Sulzfeld, Germany) received either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Jennewein 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 0.41% 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 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 data, the mean intake of the HMO mixture ranged from 5.01 to 6.88 g/kg/day for male rats and 6.26 to 7.91 g/kg/day for the female rats. This resulted in a mean intake of 6'-SL of 0.20 to 0.28 g/kg/day in males and 0.25 to 0.32 g/kg/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 ± 0.38 x 10^3 vs 0.80 ± 0.2 x 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 - 12.81 x 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 x 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 (≤ 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 4.0% 6'-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/day for male rats and 6.97 g/kg/day for the female rats. This resulted in a mean intake of 6'-SL of 0.23 g/kg/day in males and 0.28 g/kg/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 6'-SL and the subchronic rat dietary toxicity study conducted by Parschat et al. (2020) using a mixture of HMOs that contained 6'-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**					
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**	
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
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	-	-
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 included gastrointestinal mucosal gland dilation/inflammation or subacute inflammation, bacteria (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 evaluated were comparable to concurrent controls and unaffected by treatment with Oligosaccharide Blend. Body weight data are illustrated in Figure 2 and summarized in Table 19.

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 20). 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) (Table 21). This difference was not dose-dependent and considered unrelated to treatment. 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.

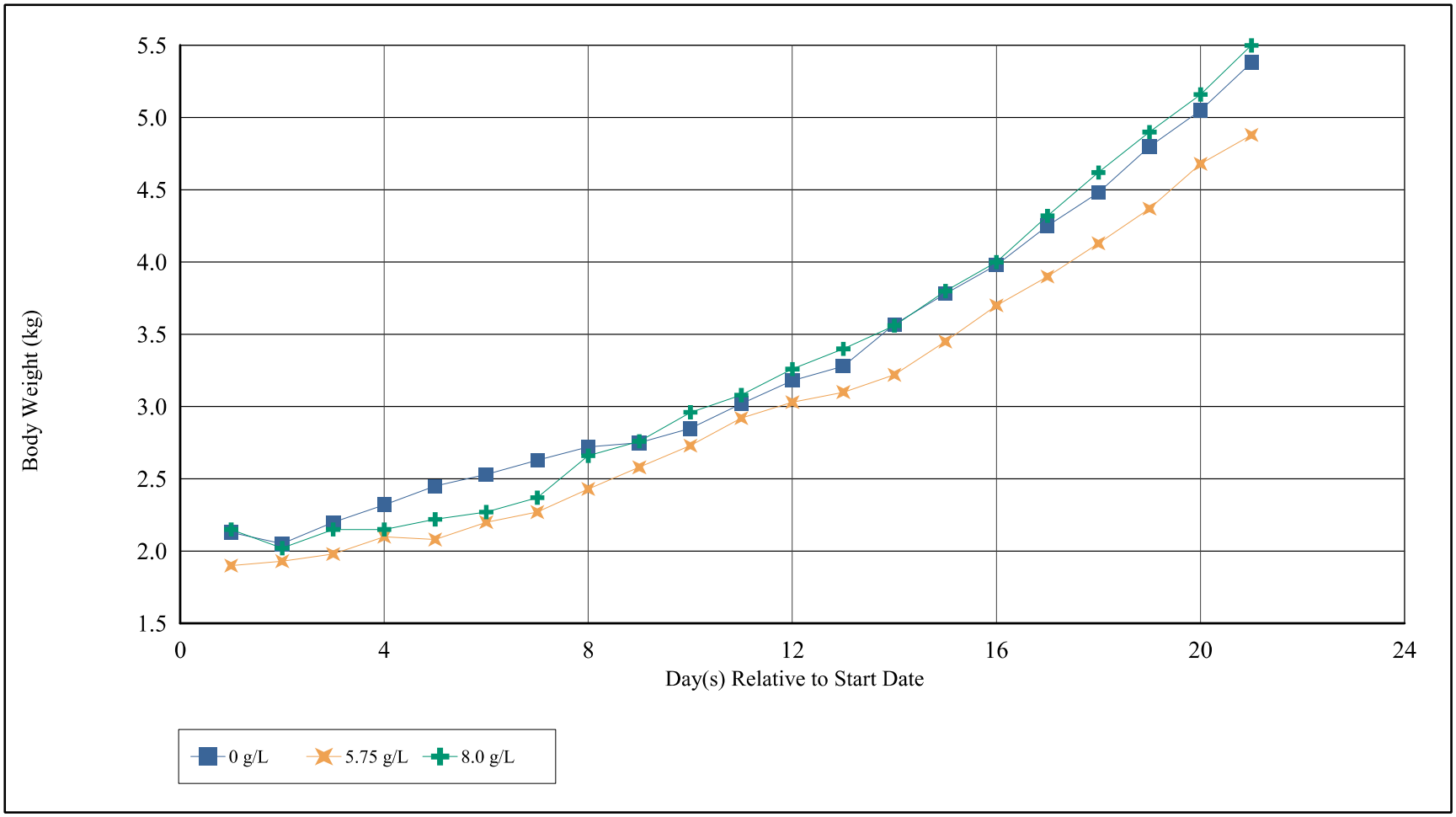


Figure 2a. Mean Body Weight Values (Male)

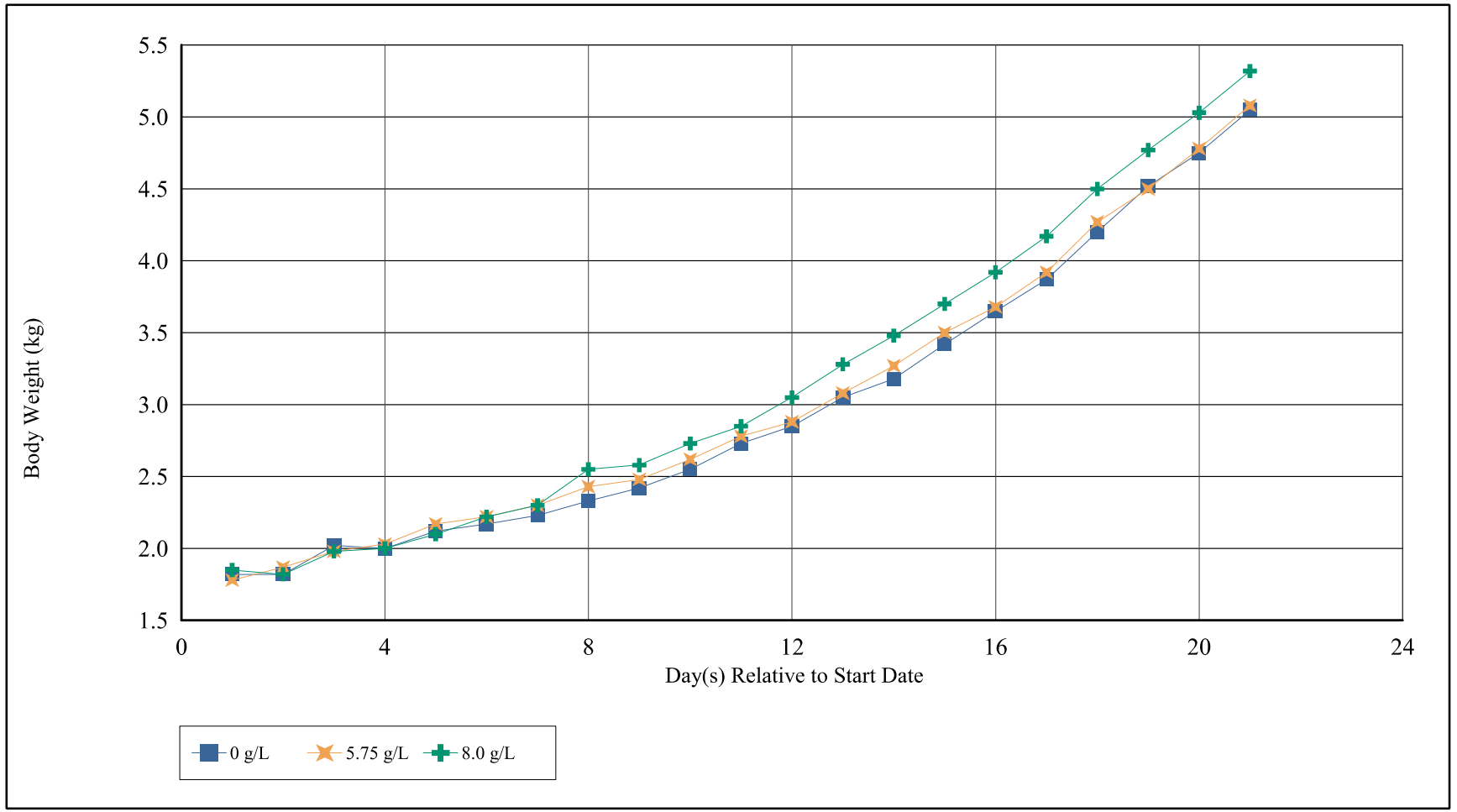


Figure 2b. Mean Body Weight Values (Female)

Table 19. Mean Body Weight Values (kg)

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

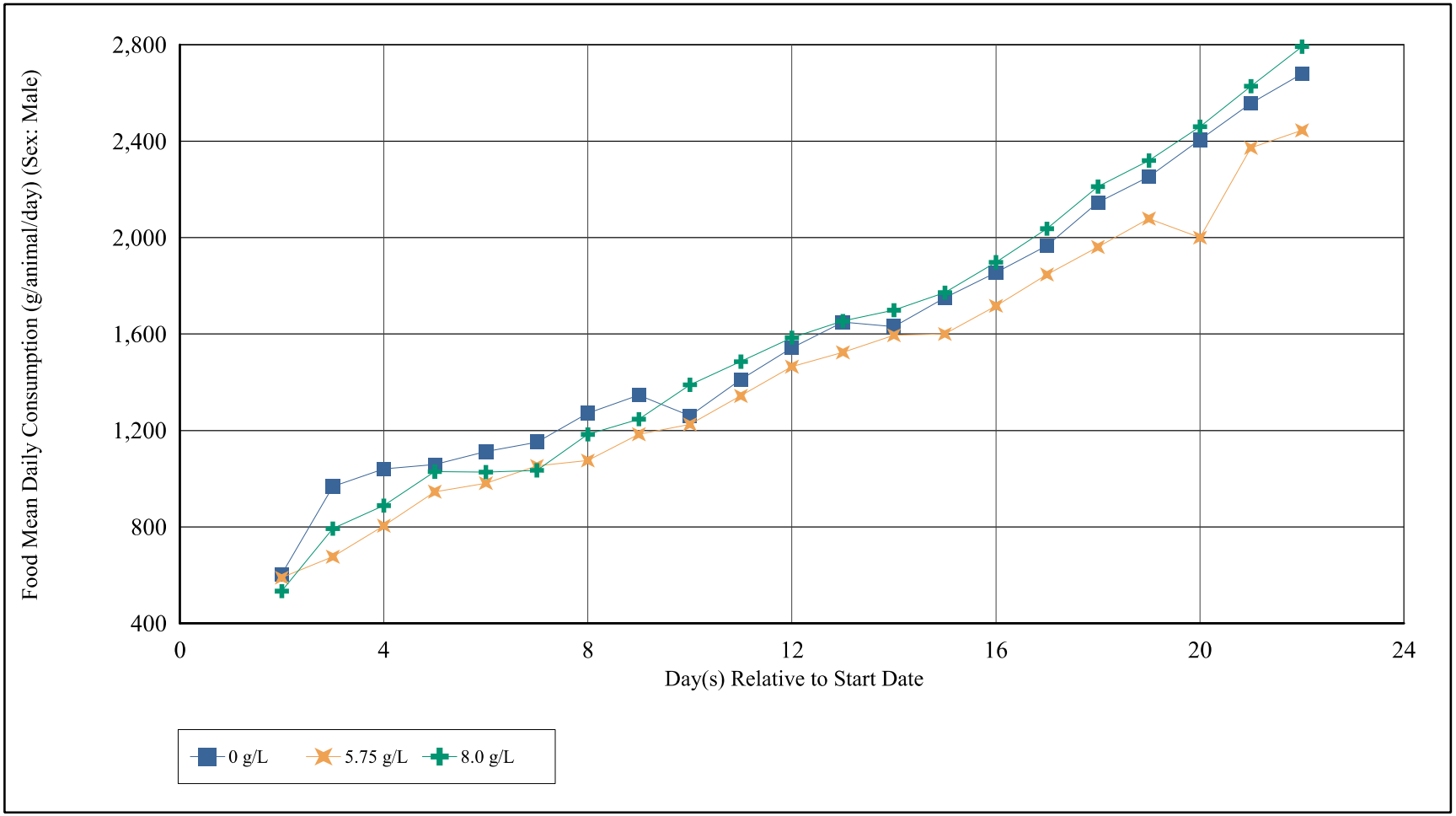


Figure 3a. Mean Feed Consumption Values (Male)

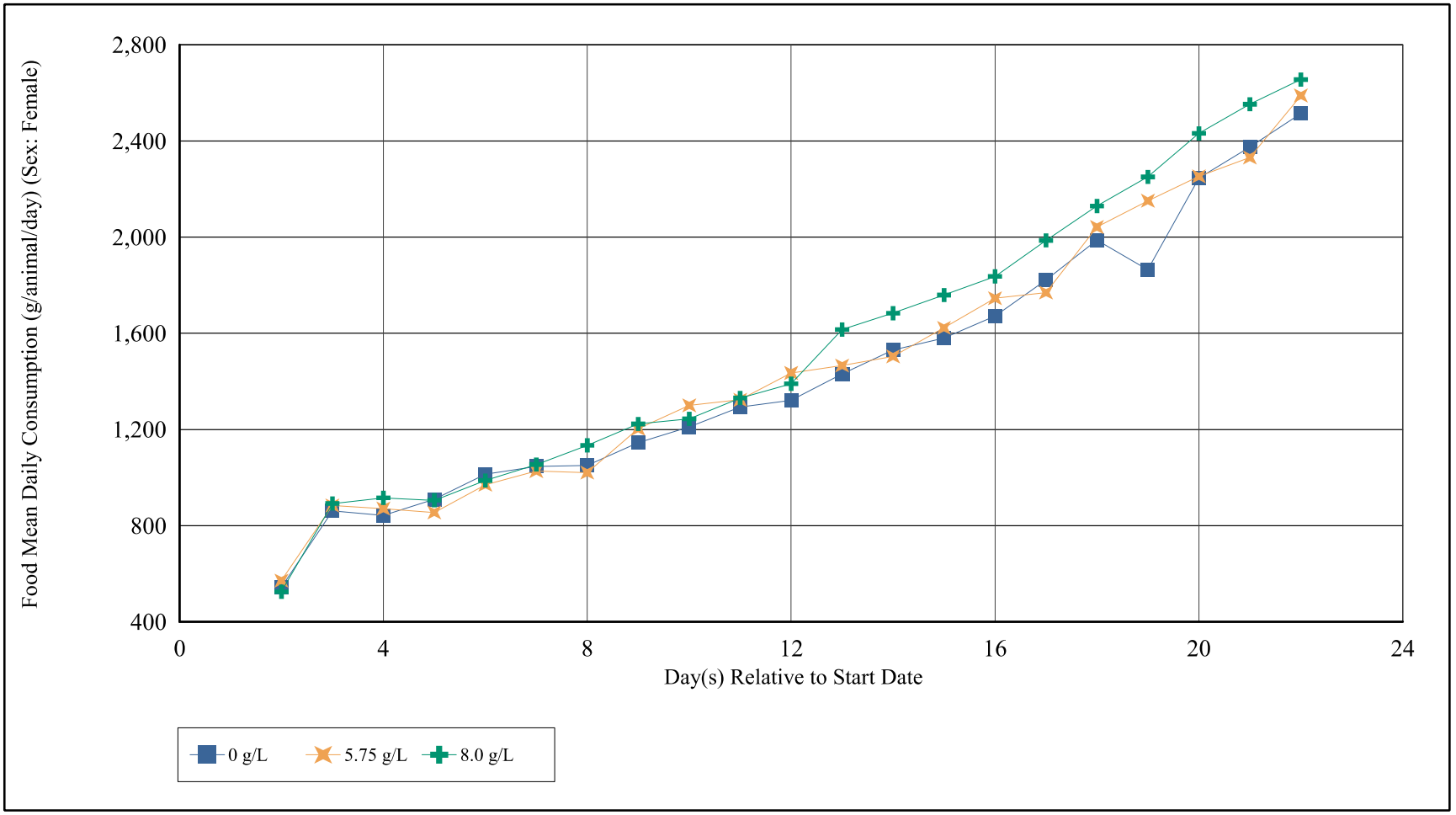


Figure 3b. Mean Feed Consumption Values (Female)

Table 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)
Leukocytes (10^3 cells/µL)	7 [g2]	0.118 ± 0.1251 (6)	0.112 ± 0.1192 (6)	0.085 ± 0.0850 (6)	0.095 ± 0.0843 (6)	0.057 ± 0.0493 (6)	0.110 ± 0.0555 (6)
	21 [g]	0.167 ± 0.1138 (6)	0.143 ± 0.1141 (6)	0.102 ± 0.1119 (5)	0.163 ± 0.1188 (6)	0.105 ± 0.0524 (6)	0.212 ± 0.0531 (6)
Erythrocytes (10^6 cells/µL)	7 [g2]	0.032 ± 0.0299 (6)	0.017 ± 0.0052 (6)	0.027 ± 0.0320 (6)	0.033 ± 0.0121 (6)	0.022 ± 0.0075 (6)	0.030 ± 0.0089 (6)
	21 [g]	0.065 ± 0.0493 (6)	0.045 ± 0.0362 (6)	0.040 ± 0.0381 (5)	0.037 ± 0.0250 (6)	0.030 ± 0.0268 (6)	0.142 ± 0.2160 (6)
Hemoglobin (g/dL)	7 [g]	0.110 ± 0.0438 (6)	0.100 ± 0.0322 (6)	0.088 ± 0.0397 (6)	0.118 ± 0.0605 (6)	0.067 ± 0.0301 (6)	0.075 ± 0.0288 (6)
	21 [g]	0.082 ± 0.0618 (6)	0.090 ± 0.0322 (6)	0.070 ± 0.0592 (5)	0.085 ± 0.0748 (6)	0.098 ± 0.0752 (6)	0.127 ± 0.0516 (6)
Hematocrit (%)	7 [g]	16.53 ± 0.339 (6)	17.35 ± 0.804 (6)	16.80 ± 0.921 (6)	16.62 ± 1.160 (6)	16.98 ± 1.350 (6)	16.47 ± 0.747 (6)
	21 [g]	17.97 ± 0.612 (6)	18.67 ± 0.480 (6)	18.20 ± 0.797 (5)	18.17 ± 0.388 (6)	18.63 ± 0.327 (6)	18.53 ± 0.999 (6)

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

[g] – ANOVA & Dunnett (Log)

[g1] – ANOVA & Dunnett

[g2] – Kruskal-Wallis & Dunn

Table 23. Coagulation Parameters (Mean ± St Dev (n))							
		Male					
Parameter	**Day**	**0 g/L**	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 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))							
		Male			Female		
Parameter	**Day**	**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.

E. CORROBORATIVE ANIMAL STUDIES

Additional neonatal piglet studies have further corroborated the safety of the consumption of 6'-SL (Jacobi et al., 2016; Obelitz-Ryom et al., 2018; Monaco et al., 2018; 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 6'-SL supplementation. Only the endpoints relevant to the safety and tolerability of sialyllactose and 6'-SL supplementation are briefly summarized below.

Jacobi et al. (2016) fed day old piglets diets containing 0, 2, or 4 g 6'-SL three times daily for 21 days to determine if different isomers of sialyllactose affects brain sialyllactose levels and modulates the microbiome. 6'-SL did not affect feed intake, growth or fecal consistency.

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.

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.

Wang et al. (2019) performed a study using sow replacement milk supplemented with a combination of 7.6 g/kg 3'-SL and 1.9 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 intact unpasteurized Jersey cow's milk supplemented with either lactose or 4.5% sialyllactose (a 6:1 ratio of 3'-SL and 6'-SL) for 19 days and assessed cognitive performance. No adverse events related to the experimental diet were reported in the study.

F. CLINICAL STUDIES

A literature search conducted through February 25, 2020 for "6-Sialyllactose" revealed no studies where 6'-SL was administered to human subjects.

G. 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-6SL* in the finished ingredient (see Section II.G). Moreover, the genes used to engineer *JBT-6SL* are not derived from major allergens and full-length FASTA alignments of amino acid sequences of the genes used to engineer *JBT-6SL* 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 28). Thus, although the protein specification does not completely eliminate the possibility that consumers of Jennewein's 6'-SL-containing ingredient may be exposed to the protein residues derived from the production organism (specification of ≤ 0.01 % protein), allergic reactions resulting from the exposure to theoretically possible protein residues derived from *JBT-6SL* in the finished ingredient are not expected.

Table 28. Percent Identity of the Genetic Manipulations in *JBT-6SL* 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
α2,6-sialylltransferase	*Streptococcus suis*	None
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.

H. REGULATORY APPROVALS ACROSS THE WORLD

6'-SL is considered GRAS in the United States and is the subject of a GRAS notification (GRN 881). Glycom's 6'-SL GRN 881, 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 0.4 g/L, 0.3 g/L in follow-on formula and infant-specific beverages, up to 5 g/kg in other foods and beverages, and up to 10 g/kg in foods for special dietary use.

VII. SUPPORTING DATA AND INFORMATION

A. REFERENCES

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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 6'-Sialyllactose Sodium Salt (6'-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 6'-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 6'-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 90% 6'-SL dry weight.

    a. 6'-Sialyllactose is a naturally occurring acidic oligosaccharide in human milk.

    b. The 6'-SL that is the subject of this GRAS Notice is structurally identical to the 6'-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 when stored from the date of production under ambient conditions.

2. Human milk oligosaccharides, including 6'-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 6'-SL in breast milk ranges from 0.1 to 0.8 g/L with means and medians ranging from 0.08 to 0.65 g/L and 0 to 1.1 g/L, respectively.

4. Genotoxicology and subchronic toxicology studies published by Phipps et al. (2019) show that 6'-SL is not genotoxic and has a no observed adverse effect level (NOAEL) of 5 g/kg bw/day, which was the highest dose tested.

5. The addition of 0.4 g/L 6'-SL in infant formula will result in mean and 90th percentile estimated daily intakes (EDI) of 0.49 g/day (70.5 mg/kg bw/day) and 1.02 g/day (143.0 mg/kg bw/day) for 0 to 6 month-old infants, which represents the maximum level of intake because infants at this age consume infant formula as a sole source of nutrition.

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

a. Published studies that quantitate the levels of 6'-SL in human milk;

b. Analytical data demonstrating that the 6'-SL produced by Jennewein is structurally identical to 6'-SL from human milk;

c. Data demonstrating the qualitative and quantitative similarities between the subject of this GRAS Notice and the 6'-SL ingredient tested by Phipps et al. (2018), which is the subject of GRN 881;

d. Corroborative published genotoxicology and 90-day subchronic toxicology studies conducted with 6'-SL or a mixture of human milk oligosaccharides containing 4.0 % of Jennewein-manufactured 6'-SL.

e. A corroborative unpublished tolerance study in neonatal piglets conducted with a mixture of HMOs containing up to 0.34 g/L of Jennewein's 6'-SL ingredient that showed an HMO mixture containing 6'-SL was well-tolerated and supported normal growth in neonatal piglets.

Therefore, 6'-SL is safe and GRAS at the proposed level of addition to the intended non-exempt, term infant formula. 6'-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
GRAS Expert Panel Member
Harvard School of Public Health

Signature: [redacted]

Date: March 19, 2020

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

Signature: [redacted]

Date: March 19, 2020

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel

Signature [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
000922	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	
Organization *(if applicable)*: Jennewein Biotechnologie GmbH			
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

6'-Sialyllactose Sodium Salt (6'-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)* GRN 881

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 Biotechnologie intends to use 6'-SL sodium salt as a substitute for other forms of 6'-SL in cow's milk-based, non-exempt infant formula for term infants at a level of 0.4 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 6'-Sialyllactose Sodium Salt (6'-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 12:44:30 -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 6'-SL GRAS Final to FDA.pdf	Submission
	References	Submission

November 7, 2020

Richard Bonnette
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration
5001 Campus Drive, HFS-225
College Park, MD 20740

RE: Questions Regarding GRN 000922

Dear Mr. Bonnette:

In response to your email dated November 2, 2020, please find our responses to your request for additional information below. We hope that our responses adequately address your requests for additional information. If you have any additional questions or require any additional clarifications, please do not hesitate to contact me at dconze@spherixgroup.com.

Sincerely,

Dietrich B. Conze, Ph.D.
Managing Partner

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

FDA's questions regarding GRN 000922 are in italicized text and our responses are in plain text.

Chemistry:

1. *In the notice, the CAS registry number cited (35890-39-2) is for 6'-sialyllactose. However, the subject of the notice is the sodium salt of 6'-sialyllactose, which has the CAS registry number 157574-76-0. Please clarify this discrepancy.*

   The correct CAS registry number for the 6'-sialyllactose sodium salt that is the subject of GRN 000922 is 157574-76-0.

Microbiology:

2. *Please state whether Escherichia coli BL21(DE3) strain "JBT-6SL" has been deposited in a recognized culture collection and provide the non-trade name designation. If the strain is not deposited, describe how the source was verified and identified.*

   The strain has been deposited at DSMZ - German Collection of Microorganisms and Cell Cultures GmbH with the deposition number DSM 33493. The host strain from which JBT-6SL was generated was purchased from a commercial source with the genotype F^- *ompT hsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3). The identity of the genetically modified strain has been verified by its susceptibility and resistance to antibiotics, the presence of the genes that have been inserted via polymerase chain reaction, and its ability to produce 6SL.

3. *Please state whether E. coli BL21(DE3) strain "JBT-6SL" is non-pathogenic and non- toxigenic.*

   JBT-6SL is non-pathogenic and non-toxigenic.

4. *The notifier 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-6SL" has the same virulence profile.*

   Jennewein engineered JBT-6SL with genes that do not encode invasion factors, adhesion molecules and enterotoxins associated with virulence using site-specific homologous recombination or transposition. Therefore, JBT-6SL has the same virulence profile as *E. coli* BL21(DE3).

5. *The notifier states that E. coli BL21(DE3) is not expected to result in any safety concerns; please state whether E. coli BL21(DE3) strain "JBT-6SL" is expected to result in any safety concerns.*

   JBT-6SL is not expected to result in any safety concerns.

6. *Please state whether E. coli BL21(DE3) strain "JBT-6SL" is capable of DNA transfer to other organisms.*

   E. coli BL21(DE3) is not able to transfer DNA to other organisms and during the engineering of JBT-6SL, Jennewein inserted genetic elements that do not confer the ability to transfer DNA to other organisms using site-specific homologous recombination or transposition. Therefore, JBT-6SL is not capable of DNA transfer to other organisms.

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

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

8. *The notifier states that the method used to detect coliforms and Enterobacteriaceae, is ISO 21528-1, which corresponds to Microbiology of the Food Chain – Horizontal Method for the Detection and Enumeration of Enterobacteriaceae – Part 1: Detection of Enterobacteriaceae. Please clarify whether this method is used in the detection of coliforms or the Enterobacteriaceae.*

   There was an error in the Notice. The correct method is ISO 21528-2, which enumerates *Enterobacteriaceae.*

Toxicology:

9. *On page 22, the notifier states that sialic acid is one of major degradation product of 6'- SL. Additionally, in Table 8 (page 23), the notifier's specifications for sialic acid are significantly higher than the specifications for the 6'-SL in GRN 881 to which the notified ingredient is being compared. Please provide a safety narrative that discusses why the sialic acid cleaved from 6'-SL via microbial and intestinal neuraminidases combined with the sialic acid present as a byproduct of your 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; Martín-Sosa et al., 2003; Oriquat et al., 2011; Martín-Sosa et al., 2004; Qiao et al., 2013). Free

sialic acid is also GRAS for use in infant formula at 50 mg/L (GRN 602, 2016). Although sialic acid is a major degradation product of 6'-SL and the specifications for sialic acid are significantly higher than the specification in GRN 000881, the sialic acid levels in Jennewein's product are below the limit of quantitation for the assay, which is 20-fold below the specification of not more than 10% (percent area) (see Table 3 in Notice). On a dry weight basis, the sialic acid content lies also below the limit of detection of 0.125 % DW (see Table below).

Table 1. Actual Levels of Sialic Acid in Jennewein 6'-SL[1]

Lot Number	Sialic Acid (% DW)
11020039	< LOD[2]
11020049	< LOD[2]
11020059	< LOD[2]
11020069	< LOD[2]
11021019	< LOD[2]

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.
[2]Limit of Detection (LOD) = 0.125 % DW

If infants consume one liter of 6'-SL-containing infant formula/day and the free sialic acid impurity is present at the limit of detection of 0.125% on a dry weight basis, the resulting intake of the free sialic acid impurity will be 0.5 mg/day (0.125% multiplied by the intended use level of 0.4 g 6'-SL/L). If the sialic acid impurity is present at the specification of 10% (% area basis), the maximum intake of the impurity would be approximately 40 mg/day. Importantly, both the actual and maximum sialic acid impurity intakes are 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 6'-SL is based on the levels of 6'-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 6'-SL via microbial and intestinal neuraminidases combined with the sialic acid impurity in the ingredient will be equivalent to what is ingested with human milk and thus not a safety concern.

References

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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. (2003). Sialyloligosaccharides in human and bovine milk and in infant formulas: variations with the progression of lactation. J Dairy Sci *86*, 52–59.

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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.

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