Spherix Consulting Group

March 20, 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 2'-Fucosyllactose (2'-FL) in Non-Exempt Term Infant Formula constitutes a new notification because Jennewein Biotechnologie GmbH intends to increase the use levels of 2'-FL in non-exempt term infant formula from 2.0 g/L to 3.64 g/L.

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 2'-Fucosyllactose (2'-FL) in Non-Exempt Term Infant Formula, and all references

Spherix Consulting Group, Inc.
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301-557-0375; info@spherixgroup.com

GRAS Determination for the Use of 2'-Fucosyllactose (2'-FL) in Non-Exempt Term Infant Formula

Prepared for:

Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

Prepared by:

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USA

March 19, 2020

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LIST OF ABBREVIATIONS

2'-FL: 2'-Fucosyllactose

3-FL: 3-Fucosyllactose

3'-SL: 3'-Sialyllactose

6'-SL: 6'-Sialyllactose

Alb: Albumin

ALT: Alanine aminotransferase

araA: Arabinose isomerase

BMI: Body mass index

BW: Body weight

CBPI: Cytokinesis-block proliferation index

CFR: United States Code of Federal Regulations

CFU: Colony forming units

CHO: Chinese hamster ovary cells

CI: Confidence interval

COSY: Correlation spectroscopy

DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen

DW: Dry weight

EDI: Estimated daily intake

EFSA: European Food Safety Authority

EU: Endotoxin unit

F6PPK: Fructose-6-phosphate phosphoketolase

FCC: Food Chemicals Codex

FDA: United States Food and Drug Administration

FFDCA: Federal Food, Drug, and Cosmetic Act

FOIA: Freedom of information Act

FOS: Fructooligosaccharides

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

Fru-6-P: Fructose-6-phosphate

FSSC: Food Safety System Certification

FUT: Fucosyltransferase

GI: Gastrointestinal

Glc-1-P: Glucose-1-phosphate

Glc-6-P: Glucose-6-phosphate

Gln-1-P: Glucosamine-1-phosphate

Gln-6-P: Glucosamine-6-phosphate

Glob: Gobulin

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

GMO: Genetically modified organism

GMP: Good manufacturing practices

GOS: Galactooligosaccharides

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

HCD: Historical control data

HDL-C: High-density lipoprotein cholesterol

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

HMO: Human milk oligosaccharides

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

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

ICP-MS: Inductively coupled plasma mass spectrometry

IFNγ: Interferon gamma

LC-MS: Liquid chromatography coupled with mass spectrometry

LDL-C: Low-density lipoprotein cholesterol

LDPE: Low-density polyethylene

LNDFHI: Lacto-*N*-difucohexaose I

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

LNT: Lacto-N-tetraose

LOD: Limit of detection

LOQ: Limit of quantitation

MCH: Mean corpuscular hemoglobin

MCV: Mean corpuscular volume

ND: Not detected

NHANES: National Health and Nutrition Examination Surveys

NIH: National Institutes of Health

NMR: Nuclear magnetic resonance

NOAEL: No Observed Adverse Effect Level

OECD: Organization for Economic Cooperation and Development

PCR: Polymerase chain reaction

Ph Eur: European Pharmacopoeia

pLNnH: para-lacto-*N*-neohexaose

qPCR: Quantitative polymerase chain reaction

RI: Replicative index

TP: Total protein

UDP-Gal: UDP-galactose

UDP-Glc: UDP-glucose

UDP-GlcNAc: UDP-*N*-acetylglucosamine

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

A. SUBMISSION OF GRAS NOTICE

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

B. NAME AND ADDRESS OF THE SPONSOR

Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

C. COMMON OR USUAL NAME

2'-Fucosyllactose (2'-FL)

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

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

E. INTENDED USE

Jennewein intends to use 2'-FL as an ingredient in cow's milk-based, non-exempt term infant formula.

F. BASIS FOR GRAS DETERMINATION

This GRAS determination for the use of 2'-FL 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 2'-FL 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 2'-FL 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 % 2'-FL dry weight.

    a. 2'-Fucosyllactose is a neutral, fucosylated oligosaccharide in human milk.

    b. The 2'-FL that is the subject of this GRAS Notice is structurally identical to the 2'-FL present in human breast milk.

    c. The subject of this GRAS Notice is also the subject of GRN 571 and the supplement to GRN 571, both of which received "no questions" letters from the United States Food and Drug Administration.

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

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

    f. 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).

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

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

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

3. Published studies show that the amount of 2'-FL in breast milk ranges from 0 to 13.8 g/L, with means and medians ranging from 0.01 to 4.6 and 0.01 to 5.2 g/L, respectively.

4. Additional genotoxicology and subchronic toxicology studies published and/or conducted since the filing of GRN 571 show that 2'-FL is not genotoxic and has a No Observed Adverse Effect Level (NOAEL) of 5 g/kg/day in rats and 0.29 g/kg/day in neonatal piglets.

5. The addition of 3.64 g/L 2'-FL in infant formula will result in mean and 90th percentile intakes of 3.3 g/day (0.52 mg/kg/day) and 4.6 g/day (0.56 g/kg bw/day), respectively, for infants 0 to 5 months-old and 0.29 g/day (0.32 mg/kg/day) and 4.0 g/day (0.36 g/kg bw/day) for infants 6 to 11 months-old.

6. The safety of exposure to Jennewein's 2'-FL ingredient at its intended use level is supported by:

    a. Published studies that quantitate the levels of 2'-FL in human milk;

    b. Analytical data demonstrating that the 2'-FL produced by Jennewein is structurally identical to 2'-FL from human milk;

    c. The published and unpublished genotoxicology and subchronic toxicology studies showing that 2'-FL is not genotoxic and has a No Observed Adverse Effect Level (NOAEL) of 5 g/kg/day in rats.

    d. Corroborative unpublished and published tolerance studies in neonatal piglets showing that up to 3.92 g/L of Jennewein-manufactured 2'-FL alone and in the presence of other HMOs was well-tolerated and supported normal growth in neonatal piglets.

Therefore, 2'-FL is safe and GRAS at the proposed level of addition to the intended infant formula. 2'-Fucosyllactose 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] 13/3/20

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

2'-Fucosyllactose (2'-FL; CAS No. 41263-94-9)

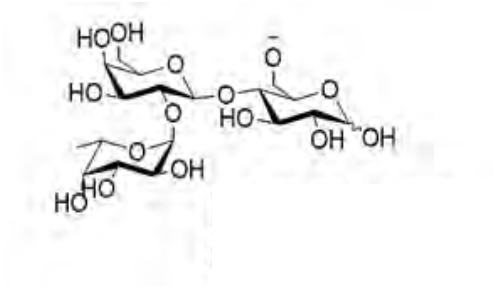
B. CHEMICAL NAME

α-L-Fucopyranosyl-(1→2)β-D-galactopyranosyl-(1→4)-D-glucopyranoside

C. MOLECULAR FORMULA AND MASS

$C_{18}H_{31}O_{15}^-$; 488.439 g/mol

D. STRUCTURAL FORMULA



E. DESCRIPTION OF 2'-FUCOSYLLACTOSE

2-Fucosyllactose (2'-FL) is a fucosylated, neutral trisaccharide composed of L-fucose, D-galactose, and D-glucose units. It is one of the most prevalent oligosaccharides in human milk (Urashima et al., 2012). The subject of this Notice is the subject of GRN 571, which received a "no questions" letter from FDA in 2015 (GRN571), and a supplement to GRN 571, which was filed with FDA in 2019, also received a "no questions" letter, and documented changes to the production organism. The spray-dried, powdered ingredient is produced by fermentation using a genetically engineered strain of *Escherichia coli* BL21(DE3), contains not less than 90% 2'-FL, and the structure of which is identical to that of the 2'-FL found in human milk.

F. PRODUCTION PROCESS

As described in the GRN 571 Supplement, 2'-FL is manufactured by fermentation using a genetically engineered strain of *E. coli* BL21(DE3). 2'-Fucosyllactose is purified from the fermentation medium and the resulting 2'-FL concentrate is spray-dried into a powder.

1. Description of the Production Strain

Because the subject of this GRAS Notice is the same as the subject of GRN 571 that summarized the genetic engineering used to generate the current production organism *E. coli* BL21(DE3) #1242, also known as *JBT-2FL*, the description of the genetic engineering provided in GRN 571 is incorporated by reference (see Appendix K, pg. 282-288). Briefly, *JBT-2FL* was engineered from the early 2'-FL production strain #742, which lacks the genes encoding a β-galactosidase, an L-arabinose-isomerase, a L-fucose isomerase, a L-fuculokinase, an *N*-acetylglucosamine 6-phosphate deacetylase, a glucosamine 6-phosphate deaminase, a lipopolysaccharide biosynthesis protein, and a UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase and expresses the genes encoding a UDP-galactose-4-epimerase, a galactosyltransferase, a galactokinase, a galactose mutarotase, a sucrose hydrolase, a sucrose transporter, a fructokinase, a transcriptional regulator, a phosphomannomutase, a mannose-1-phosphate guanosyltransferase, a GDP-mannose-4,6-dehydratase, a GDP-L-fucose synthase, and a lactose permease. An α-1,2-fucosyltransferase and a heterologous 2'-FL exporter were then integrated into strain #742 to allow for synthesis and export of 2'-FL into the culture medium. The resulting integrants were subjected to two rounds of nitrosoguanidine (NTG) mutagenesis and screened for their ability to produce high levels of 2'-FL, resulting in *JBT-2FL*. The final production stain was designated as #1242 or *JBT-2FL*.

2. Manufacturing

a. Quality

Production of 2'-FL occurs at the Jennewein Biotechnologie GmbH production facility in Maarweg 32, 53619 Rheinbreitbach, Germany, which is Food Safety System Certification (FSSC) 22000 and ISO 9001:2015 compliant, and an FDA-registered Food Facility (Registration). Production also occurs at other Jennewein-qualified manufacturers that are GMP-, ISO-, and International Featured Standards Food 6.1-compliant as shown by third-party audits.

b. Processing Aids and Food Contact Substances

All raw materials, processing aids, and food contact substances used to produce the 2'-FL powder are the same as those used to produce the 2'-FL that is the subject of GRN 571, which received a "no questions" letter from FDA. Therefore, the quality of the processing aids and raw materials and composition of the media described in GRN 571 (see pg. 17; Appendix E, pg. 99-144; Appendix J, pg. 280-281) are incorporated by reference. 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. The lactase used to degrade residual lactose in the fermentation medium complies with the recommended specifications of both the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) for food-grade enzymes (see pg. 21 of GRN 571 Supplement). 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

The 2'-FL that is the subject of this Notice is manufactured using the same process described in GRN 571 and the GRN 571 supplement. Therefore, the detailed summary of the production process provided in GRN 571 (pg. 6-9) is incorporated by reference. Briefly, 2'-FL production involves three steps (Figure 1). During Step 1, *E. coli* BL21(DE3) *JBT-2FL* is cultured in minimal medium containing a carbon source (glucose, sucrose, or glycerol, or a combination thereof) and the substrate lactose, which is present throughout the process. Fermentation of lactose results in the production and secretion of 2'-FL into the culture medium. Step 2 involves purification and concentration of 2'-FL from the culture medium. Step 3 involves spray-drying of the 2'-FL concentrate, producing powdered 2'-FL. Additionally, lactase may be added at the end of the fermentation process to degrade excess lactose in the medium and increase product yield.

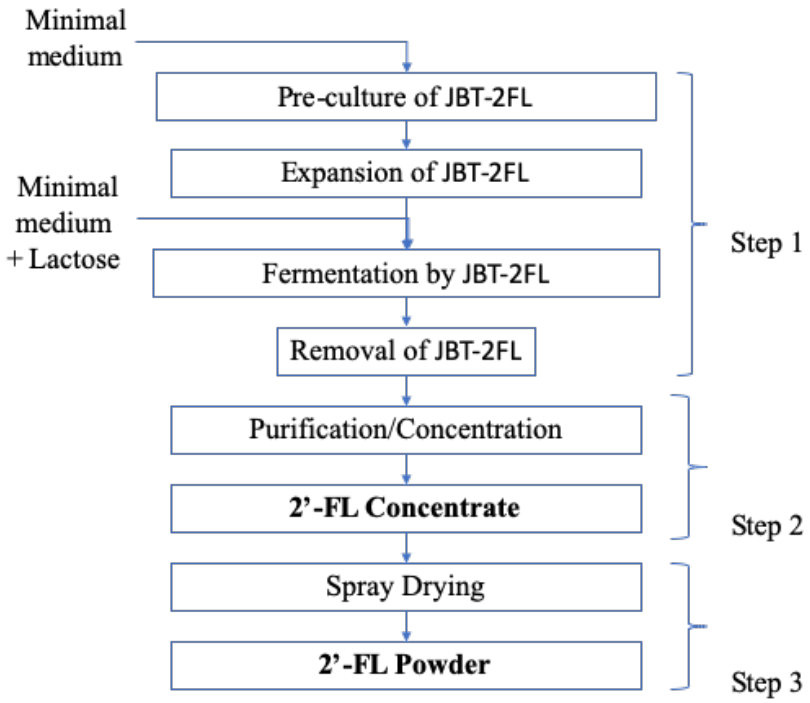


Figure 1. Production Process for 2'-Fucosyllactose.
JBT-2FL is expanded in minimal medium and with the addition of the lactose, 2'-fucosyllactose (2'-FL) is produced. The production strain/biomass is removed, yielding the oligosaccharide-containing fermentation medium. The medium is purified and concentrated in a series of filtration, ion exchange, electrodialysis, and decolorization steps to yield the 2'-FL concentrate. Finally, the concentrate is spray dried to generate powdered 2'-FL.

G. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. 2'-FL Product Specifications and Batch Data

To ensure a consistent food-grade product that is free of genetically-modified ingredients, each batch of 2'-FL manufactured with the new production strain *JBT-2FL* is evaluated against the same product specifications that were established in GRN 571. The product specifications control the amount of 2'-FL, carbohydrate by-products, DNA and endotoxin residues derived from the production strain, heavy metals, and selected microbes. Each parameter is measured using the same compendial and/or internally validated methods that were provided in GRN 571. Because batch data showing compliance with the GRN 571 specifications and were provided in the GRN 571 supplement, the batch data provided in the GRN 571 supplement are incorporated by reference (see pg. 32 of GRN 571 supplement,).

H. STABILITY

1. Genetic Stability of the Production Strain

Section 6.2 of GRN 571 (pg. 299) summarizes the stability of the genes integrated into JBT-2FL and is therefore incorporated by reference. To ensure genomic stability and finished product batch-to-batch consistency, all genetic modifications were stably integrated into the genome of JBT-2FL and the production of 2'-FL occurs in a sterile environment. Thus, the production strain is not expected to lose its ability to produce a consistent finished product.

2. Stability of 2'-Fucosyllactose

As summarized in GRN 571, the subject of this Notice is stable for at least 104 weeks (2 years) when stored at 25 °C and 60% humidity, and for not less than 26 weeks (6 months) when stored at 40 °C and 75% humidity (see Section 2.4, pg. 27 -30). 2'-Fucosyllactose is also the subject of other GRAS Notifications and stability data provided in those GRAS Notification all support a 104-week shelf-life when stored at 25 °C and 60% humidity (GRN 546; GRN 650; GRN 735; GRN 749).

To understand whether 2'-FL has a similar stability when combined with other human milk oligosaccharides, 2'-FL was mixed with 3-fucosyllactose (3-FL), lacto-*N*-tetraose (LNT), 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL), and 2'-FL stability was monitored over the course of 26 weeks under accelerated (40°C and 75% relative humidity) conditions and 52 weeks under ambient (25°C and 60% relative humidity) conditions. The mixture contained approximately 50% 2'-FL by dry weight after production and was stored in high density polyethylene bottles. Both 2'-FL and moisture content were monitored over time using the same methods that are used for batch qualification.

2'-Fucosyllactose remained relatively unchanged over the course of the 52-week testing period. Moisture content increased from 5.7% to 7.8%; however, the parameter did not exceed the product specification of not more than 9% at week 52 (Table 1).

Under accelerated conditions, 2'-FL decreased, and moisture increased over the course of the study, with moisture falling out of specification by 26 weeks (Table 2).

Together these results support a 2'-FL shelf-life of 2 years when stored alone under ambient conditions, and 1 year when mixed with 3-FL, LNT, 3'-SL, and 6'-SL and stored under ambient conditions.

Table 1. Stability of 2'-Fucosyllactose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Ambient Conditions (25°C, 60% Relative Humidity)

Batch 4011-1004303107		Moisture		2'-FL	
		%	% of baseline	% DW	% of baseline
Specification:		≤ 9	NA	NA	NA
Interval	Baseline	5.7	100.0	49.18	100
	Week 1	5.2	91.9	48.95	99.5
	Week 4	6.2	109.2	49.85	101.4
	Week 8	6.1	108.3	48.90	99.5
	Week 13	6.1	107.2	48.45	98.5
	Week 26	6.9	121.7	46.75	95.1
	Week 39	7.3	129.3	49.25	100.1
	Week 52	7.8	137.0	50.05	101.8

Abbreviations: DW, dry weight; 2'-FL, 2'-fucosyllactose; NA, not applicable.

Table 2. Stability of 2'-Fucosyllactose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Accelerated Conditions (40°C, 75% Relative Humidity)

Batch 4011-1004303107		Moisture		2'-FL	
		%	% of baseline	% DW	% of baseline
Specification:		≤ 9	NA	NA	NA
Interval	Baseline	5.7	100.0	49.18	100
	Week 1	5.8	101.4	49.05	99.7
	Week 4	6.6	117.1	49.25	100.7
	Week 8	7.3	129.1	48.95	99.5
	Week 13	8.7	153.6	48.96	98.8
	Week 26	9.9	174.6	43.90	89.3

Abbreviations: DW, dry weight; 2'-FL, 2'-fucosyllactose; NA, not applicable.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

The intended effect of adding 2'-FL to non-exempt term infant formula is to increase 2'-FL intake in formula-fed infants and promote the growth of beneficial bacteria, including, but not limited to bifidobacteria.

B. HISTORY OF EXPOSURE

2'-FL is one of the most abundant oligosaccharides in human milk. It is also found in the milk of goats, pigs, chimpanzees, bonobos, and orangutans (Castanys-Muñoz et al., 2013; Chaturvedi et al., 2001). In cow's milk, which is the most common milk used in the production of infant formula in the United States, the oligosaccharide content is 100 to 1000 times lower than human milk. Moreover, less than 1% of cow's milk oligosaccharides is fucosylated (Aldredge et al., 2013) and synthetic forms of 2'-FL have also been determined GRAS for use in infant formula and selected conventional foods and beverages (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019). Thus, humans are predominantly exposed to 2'-FL either through the ingestion of breast milk and/or products containing synthetic forms of 2'-FL.

The concentration of 2'-FL in human breast milk has been quantitated in 33 studies with greater than 5 donors (Alderete et al., 2015; Austin et al., 2016; Austin et al., 2019; Azad et al., 2018; Chaturvedi et al., 1997; Kunz et al., 2017; Larsson et al., 2019; Leo et al., 2010; Ma et al., 2018; Marx et al., 2014; McGuire et al., 2017; McJarrow et al., 2019; Nijman et al., 2018; Paganini et al., 2019; Samuel et al., 2019; Sjögren et al., 2007; Williams et al., 2017; Spevacek et al., 2015; Sprenger et al., 2017; Thurl et al., 2010; Coppa et al., 2011; Gabrielli et al., 2011; Erney et al., 2000; Nakhla et al., 1999; Asakuma et al., 2008; Chaturvedi et al., 2001; Smilowitz et al., 2013; Van Niekerk et al., 2014; Goehring et al., 2014; Hong et al., 2014). The results of 12 of these studies were summarized in a systematic review conducted by Thurl et al. (2017). A summary of the findings reported Thurl et al. (2017) and the 21 additional studies is presented in Table 3. Although the levels of 2'-FL in human milk vary with ethnicity, Secretor and Lewis-blood group status, lactation period, and term vs preterm birth, the available studies show that the concentration of 2'-FL in breast milk generally ranges from 0 to 13.8 g/L, with means and medians ranging from 0.01 to 4.6 and 0.01 to 5.2 g/L, respectively. Therefore, the background exposure to 2'-FL from human milk serves as the safe range for the use of Jennewein's 2'-FL in infant formula.

Additionally, synthetic forms of 2'-FL, including the subject of this Notice, are GRAS at levels up to 2.4 g/L in non-exempt, cow's milk-based term infant and toddler formulas, as well as in baby foods at levels ranging from 0.24 to 1.2 g/serving, and in beverages and beverage bases, dairy product analogs, infant and toddler foods including follow-on formulas, grain products and pastas, milk and milk products, and processed fruits and fruit juices at use levels ranging from 0.28 to 1.2 g/serving (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 815, 2019; GRN 852, 2019).

Table 3. Studies Determining the Concentration of 2'-Fucosyllactose (2'-FL) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	2'-FL concentration
Alderete et al., 2015	United States	37 donors	1 and 6 months	Highest median ± interquartile range: 2.7 ± 3.7 g/L (1 month) Lowest median ± interquartile range: 2.4 ± 2.8 g/L (6 month) *Only median ± interquartile ranges were reported
Austin et al., 2016	China	450 donors (approximately 90 donors/timepoint)	Days 5-11, Days 12-30, Months 1-2, Months 2-4, Months 4-8	Reported range: 0.026 – 4.9 g/L Highest mean: 2.1 ± 1.4 g/L (days 5-11) Highest median: 2.1 g/L (days 5-11) Lowest mean: 1.1 ± 0.7 g/L (4-8 months) Lowest median: 1.2 g/L (4-8 months)
Austin et al., 2019	Switzerland	27 donors with 33 preterm infants (approx. 25 samples/timepoint) 34 donors with 34 term infants (approx. 28 samples/timepoint)	Weekly for 8 weeks after delivery (preterm and term) then every 2 weeks until 16 weeks (preterm only)	Reported range: 0.07 – 6.1 g/L Highest mean: 3.2 ± 1.9 g/L (Term, week 1) Highest median: 3.4 g/L (Term, week 1) Lowest mean: 1.3 ± 1.0 g/L (Preterm, weeks 12, 14 and 16) Lowest median: 1.3 g/L (Preterm, week 14)
Azad et al., 2018	Canada	427 donors	3- 4 months postpartum	Reported range: 0 – 13.8 g/L Mean: 4.6 ± 3.8 g/L Median: 5.0 g/L
Berger et al., 2020	United States	50 donors	1 and 6 months postpartum	Reported range: 0 – 6.0 g/L
Chaturvedi et al., 1997	Mexico	50 donors	1-2 months postpartum	Mean: 1.7 ± 0.08 g/L *Only mean ± standard error was reported
Kunz et al., 2017	Spain	32 donors (21 secretors; 11 nonsecretors	Lactation days 1-7 (colostrum), 8-15 (transitional milk), and 16-30 (mature milk)	Highest median: 2.9 g/L (0 – 4.6 g/L) (Preterm, colostrum) Lowest median: 2.0 g/L (0 – 3.3 g/L) (Preterm, mature milk) *Only median and interquartile ranges were reported

Table 3. Studies Determining the Concentration of 2'-Fucosyllactose (2'-FL) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	2'-FL concentration
Larsson et al., 2019	Denmark	11 mothers with high weight infants 15 mothers with normal weight infants	5 and 9 months	Highest median: 4.1 g/L (3.4 – 5.0 g/L) (5 months; high weight group) Lowest median: 5.2 g/L (2.1 – 3.7 g/L) (9 months; normal weight group) *Only median and interquartile ranges were reported
Leo et al., 2009	Samoa	8 mothers	5-10 days and greater than 10 days postpartum	Highest mean: 0.7 ± 0.8 g/L (greater than 10 days post-partum) Lowest mean: 0.2 ± 0.8 g/L (5-10days post-partum) *Median and range was not reported
Ma et al., 2018	China, Malaysia	China: 20 donors Malaysia: 26 donors	China: days 14, 30, 60, 90, 120, 180, and 240 post-partum Malaysia: days 2, 60, 180, and 365 post-partum	<u>Chinese Mothers</u> Highest mean: 1.4 ± 1.1 g/L (30 days post-partum) Lowest mean: 0.7 ± 0.8 g/L (240 days post-partum) <u>Malaysian Mothers</u> Highest mean: 2.2 ± 1.7 g/L (2 days post-partum) Lowest mean: 0.7 ± 0.6 g/L (365 days post-partum) *Only means ± standard deviations were reported
Marx et al., 2014	United States	26 mothers with infants in the neonatal intensive care unit 31 samples of donor milk	Random	<u>Mothers milk</u> Reported range: ~0 – 8.2 g/L Median (interquartile range): ~3.8 (1.7 – 4.9) g/L <u>Donor milk</u> Reported range: ~0.1 – 9.0 g/L Median and interquartile range: ~2.2 (1.8 – 4.6) g/L *values obtained from a graph

Table 3. Studies Determining the Concentration of 2'-Fucosyllactose (2'-FL) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	2'-FL concentration
McGuire et al., 2017	Ghana, Kenya, Peru, Spain, Sweden, rural and urban Ethiopia and Gambia, Washington State (USA), and California (USA)	410 healthy women	2 weeks to 5 months postpartum	Highest mean: 3.4 ± 0.4 g/L (United States -California; n=19) Lowest mean: 0.7 ± 0.1 g/L (Ghana; n=40) *Only means ± standard deviations were reported
McJarrow et al., 2019	United Arab Emirates	Transitional milk: 41 donors Mature milk: 40 donors	Days 5-15 post-partum (transitional milk) 6 months post-partum (mature milk)	Highest mean: 2.0 ± 1.8 g/L (Transitional milk) Lowest mean: 1.0 ± 0.9 g/L (Mature milk) *Only means ± standard deviations were reported
Nijman et al., 2018	United States	10 donors	Day 3 and 42 postpartum	Highest mean: 3.8 ± 0.1 g/L (day 3) Lowest mean: 2.5 ± 0.3 g/L (day 42)
Paganini et al., 2019	Kenya	80 donors	No specific timepoint	Median (interquartile range): 0.7 (0.0-1.0) g/L *Mean and range was not reported
Samuel et al., 2019	Europe	290 donors	Days 2, 17, 30, 60, 90, and 120 of lactation	Reported range: 0.013 – 9.5 g/L Highest mean: 3.7 ± 1.9 g/L (day 2 postpartum) Highest median: 3.8 g/L (day 2 postpartum) Lowest mean: 1.6 ± 0.7 g/L (day 120 postpartum) Lowest median: 1.6 g/L (day 120 postpartum)
Sjogren et al., 2007	Sweden	11 allergic 9 non-allergic women	2-4 days postpartum	Range: 0.0 – 5.2 g/L Highest median: 3.3 g/L (allergic mothers) Lowest median: 3.0 g/L (non-allergic mothers) *Means were not reported

Table 3. Studies Determining the Concentration of 2'-Fucosyllactose (2'-FL) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	2'-FL concentration
Spevacek et al., 2015	United States	Mothers of 15 term and 13 preterm	Colostrum (1st week), transition (14 days postpartum), and mature milk (28 d postpartum)	Highest mean: 2.7 ± 2.0 g/L (Term colostrum) Lowest mean: 1.1 ± 1.2 g/L (Preterm mature milk) *Medians were not reported
Sprenger et al., 2017	Singapore	Approx 50 donors	1, 2, and 4 months postpartum	Reported range: 0.004 – 5.0 g/L Highest mean: 2.1 ± 0.8 g/L (1 month postpartum) Highest median: 2.1 g/L (1 month postpartum) Lowest mean: 0.01 ± 0.005 g/L (4 months postpartum) Lowest median: 0.01 g/L (4 months postpartum)
Thurl et al., 2017	Worldwide	Systematic review of 21 previous studies (not all reported LNT)	Lactation days 0 to >100	Highest mean: 2.8 g/L (95% confidence limit of 0.8 – 4.8; n=74 preterm mothers/230 samples) Lowest mean: 2.7 g/L (95% confidence limit of 2.4 – 3.0; n=353 term mothers/556 samples) *Medians were not reported
Williams et al., 2017	United States (Washington and Idaho)	16 donors	Weekly for 7 months (average time post-partum at enrollment 161 days)	Mean = 0.96 ± 0.15 g/L *Only one mean ± standard error was reported

C. INTENDED USES

2'-Fucosyllactose is GRAS in the United States for use in term, cow's milk-based non-exempt infant formula at levels up to 2.4 g/L reconstituted formula (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019). Jennewein's 2'-FL, in specific, is GRAS for use in term, cow's milk-based non-exempt infant formula at 2.0 g/L (GRN 571, 2015). To accommodate variations in 2'-FL levels that occur due to ethnicity, secretor and Lewis-blood group status, lactation period, and term vs preterm birth, Jennewein intends to increase the use level of its 2'-FL product from 2.0 g/L to 3.64 g/L of reconstituted formula.

D. ESTIMATED DAILY INTAKE

Because Jennewein intends to increase the use of its 2'-FL product from 2.0 to 3.64 g/L reconstituted formula, the estimated daily exposures provided in GRN 571, which were calculated using the 2009-2010 NHANES database, were multiplied by the fold increase (1.82-fold) to determine the estimated daily exposure to Jennewein's 2'FL at the increased use level (Table 4). Based on the proposed use of up to 3.64 g Jennewein 2'-FL/L in non-exempt infant formulas for term infants, the highest estimated mean intake of Jennewein 2'-FL occurs in infants of 0-5 months and is 3.3 g/d and the estimated intake at the 90th percentile is 4.6 g/d. A small number of infants consume formulas after the first year of life, and formula intakes are lower than intakes by infants 0-5 or 6-11 months of age.

Table 4. Estimated Daily Intake of Jennewein 2'-FL from Infant Formula

Population[b]	n[c]	Percent users[d]	Mean		90th Percentile	
			g/d[a]	g/kg bw/day	g/d[a]	g/kg bw/day
Infants, 0-5 mo	141	100	3.3	0.52	4.6	0.56
Infants, 6-11 mo	142	86.3	2.9	0.32	4.0	0.36

Abbreviations: 2'-FL = 2'-fucosyllactose; d =day; g = gram(s); bw = body weight; mo = month(s).
Source: NHANES 2009-2010 Data; Jennewein Biotechnologie, GmbH.
[a]Values are 1.82-fold those noted in Table 1 of GRN 571.
[b]Breastfeeding infants and children were excluded from the sample population.
[c]Number of people consuming infant formula during the study period.
[d]Weighted percent.
Note: Because this is a daily average, some participants who had day 1 but not day 2 data are included using a single day of consumption.

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 2'-FL according to the specified conditions of use in non-exempt term infant formula is based on the following: the published studies that quantitate the levels of 2'-FL in human milk (see Section III.B); the analytical data demonstrating that the 2'-FL produced by Jennewein is structurally identical to 2'-FL from human milk; published and unpublished toxicological studies summarized in previous GRAS Notifications; and the GRAS status of the subject of this Notice (GRN 546, GRN 571, GRN 650, GRN 735, GRN 749; GRN 571 Supplement).

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

Fucosylated neutral HMOs constitute 35-50% of the HMOs in breast milk of which 2'-fucosyllactose is the most abundant (reviewed in Vandenplas et al., 2018; Thurl et al., 2017). Although its absolute concentration varies with ethnicity, secretor and Lewis-blood group status, 2'-FL levels are generally higher in colostrum and lower in mature breast milk (Erney et al., 2000). Based on available data, the levels of 2'-FL in breast milk range from 0 to 13.8 g/L with means and medians ranging from 0.01 to 4.6 and 0.01 to 5.2 g/L, respectively (See Section III.B). Thus, Jennewein's intended use of 3.64 g 2'-FL/L infant formula is well within the established range of 2'-FL 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 2'-FL will not pose risks to infants consuming formula containing 2'-FL.

In the United States, eight 2'-FL-containing ingredients have been determined GRAS for use in non-exempt term infant formulas (GRN 546; GRN 571; GRN 650; GRN 735; GRN 749; GRN 815; GRN 852). The first 2'-FL product to be determined GRAS is the subject of GRN 546. It is chemically synthesized and structurally identical to the 2'-FL found in breast milk. Additionally, its safe use as an ingredient in infant formulas and selected conventional foods is supported by use levels that approximate the average 2'-FL levels found in breast milk (2.4 g/L) and a variety of published genotoxicity and subchronic toxicity studies in rats (Coulet et al., 2014). In 2015, GRN 546 received a "no questions" letter from the United States Food and Drug Administration. Since then, seven additional GRAS Notices have been filed, six are for 2'-FL products and one is for a product containing a mixture of 2'-FL and difucosyllactose. For all 2'-FL ingredients with GRAS Notices filed since GRN 546, the intended uses and use levels are similar to those specified in GRN 546. Additionally, the general recognition of safety relies on

published studies that quantitate the levels of 2'-FL in human milk, published and unpublished toxicological studies conducted either on the subject of the Notice or on the subject of another 2'-FL Notice, as well as corroborative clinical studies showing that 2'-FL is well tolerated in infants and adults. Except for GRN 859, which FDA ceased to evaluate at the notifier's request due to major deficiencies, the remaining six GRAS Notices all received "no questions" letters from the Agency. Importantly, the subject of this Notice is the subject of GRN 571, a supplement to GRN 571, and manufactured by Jennewein. Jennewein intends to increase the use level its 2'FL in infant formula from 2.0 g/L to 3.64 g/L based on a review of the publicly available studies that have quantitated the levels of 2'-FL in human milk and been published since the filing of GRN 571. Consistent with the reviews provided in previous 2'-FL GRAS Notices, 2'-FL levels in breast milk are highly variable and depends on a variety of factors including ethnicity, Secretor and Lewis-blood group status, and lactation period. The updated review now shows that the range of 2'-FL level in breast milk is greater than what was reported in GRN 546. Thus, the proposed increase in use level in infant formula will accommodate the variations in breast milk 2'-FL levels resulting from ethnicity, secretor and Lewis-blood group status, lactation period, and term vs preterm birth.

Based on these data, there is reasonable certainty that the use of Jennewein's 2'-FL per the intended use and use level is of no harm to consumers. Jennewein's 2'-FL 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 ADME of HMOs has been extensively summarized in previous GRAS Notices and opinions published by worldwide authoritative bodies (GRN 484, 2014; GRN 546, 2015; GRN 547, 2014; GRN 571, 2015; GRN 650, 2016; GRN 659, 2016; GRN 735, 2018; GRN 749, 2018; GRN 749, 2018; GRN 766, 2018; GRN 815, 2019; GRN 833, 2019; EFSA Panel on Dietetic Products, 2015; EFSA Panel on Nutrition et al., 2019). Briefly, HMOs, including 2'-FL, are highly resistant to the digestive enzymes of the gastrointestinal (GI) tract and only small amounts are absorbed intact. *In vitro* studies have shown that <5% of ingested HMOs is digested. *In vivo* studies among infants and in rats have reported that 1 to 2% of the total amount of ingested HMO is excreted unchanged in urine and the remaining unabsorbed oligosaccharides then pass through the gastrointestinal tract where it is either fermented by the select resident microbiota or excreted unchanged in the feces (Goehring et al., 2014; Ruhaak et al., 2014; Santos-Fandila et al., 2014; Dotz et al., 2014; Obermeier et al., 1999; Rudloff et al., 2012; Rudloff et al., 2006; Rudloff and Kunz, 2012; Rudloff et al., 1996; Chaturvedi et al., 2001; Gnoth et al., 2000; Engfer et al., 2000; Brand-Miller et al., 1998). Although the exact mechanisms by which HMO absorption occurs have not been fully elucidated, data from *in vitro* studies using the Caco-2 human intestinal epithelial cell model suggest that neutral HMOs are transported across the intestinal epithelium by receptor-mediated transcytosis as well as by paracellular transport, whereas acidic HMO are absorbed via the non-specific paracellular transport only (Gnoth et al., 2000).

For 2'FL specifically, two studies that evaluated its ADME have been published since the filing of GRN 571, Vazquez et al. (2017) and Marriage et al. (2015). Vazquez et al. (2017) evaluated the kinetics and metabolic fate of absorbed 2'-FL in rats. 2'-Fucosyllactose (0.2, 1.0, or 5 g/kg) was administered by gavage and LNnT, lactose, and fucose, sialic acid, 2'-FL, 6'-SL, and 3'-SL levels were quantitated in serum and urine over the course of 300 minutes by ultra-performance liquid chromatography coupled mass spectrometry (LC-MS). 0.045 +/- 0.2 μg 2'-FL/ml serum was detected in 13% of the animals at baseline. Following gavage, serum 2'-FL levels increased to a maximum of approximately 7 μg/ml 60 minutes after the administration of 0.2 g 2'-FL/kg, 20 μg/ml 180 minutes after the administration of 1 g 2'-FL/kg, and 45 μg/ml 60 minutes after the administration of 5 g 2'-FL/kg. After the maximum concentrations were reached following the administration of 0.2 and 1 g 2'-FL/kg, the levels then declined, but did not reach baseline by 300 minutes. Once the maximum concentration was reached after the administration of 45 μg/ml, the serum levels remained constant over the following 240 minutes. Additionally, the levels of the parent sugars of 2'-FL, lactose and fucose increased in plasma after the administration of 2'-FL, particularly following the administration of 5 g/kg. Urinalysis revealed that the 2'-FL levels were low at baseline and increased dose-dependently between 90 and 120 min post gavage. Additionally, urinary levels of lactose and fucose also increased after the 2'-FL gavage. Together, these data confirm the results of previous studies that small amounts of 2'-FL are absorbed and metabolized at least in part to lactose either prior to and/or after absorption.

As summarized on p. 37 of GRN 650, Marriage et al. (2015) conducted a prospective, randomized, placebo-controlled, double-blind study in infants to examine growth and tolerance of infant formulas having a caloric density approximating human milk supplemented with chemically synthesized 2'-FL, as well as the absorption of 2'-FL. Infants were enrolled within Day of Life (DOL) 5 and consumed either a standard, milk-based, commercially-available infant formula containing 2.4 g galactooligosaccharides (GOS), a standard formula supplemented with 0.2 g 2'-FL/L and 2.2 g GOS/L, a standard infant formula supplemented with 1.0 g 2'-FL/L and 1.4 g GOS/L, or breast milk from their mothers for 4 months. All formulas had a caloric density of 64.3 kcal/dL, which is comparable to human milk. 2'-Fucosyllactose absorption was measured by the levels of 2'-FL in infant plasma and urine in a subset of infants at Day of Life 42 and 119 and from the human milk of the breast-feeding mothers at Day of Life 42. Growth was measured using weight, length, and head circumference. Tolerance was measured by average stool consistency, number of stools per day, and percent of feedings associated with spit- up or vomit. The growth and tolerance results are discussed in Chapter VI, Section F.1 of this Notice.

Three hundred thirty-eight infants completed the study, 304 of whom completed the study on the assigned feeding or human milk. The number of premature discontinuations on the

study formulas was not different among the formula-fed groups. No 2'-FL was detected in the plasma of infants fed the standard milk-based commercial formula containing GOS, whereas 2'-FL was detected in the plasma and urine of infants provided the 2'-FL-supplemented formula and in infants consuming human milk, with the greatest mean 2'-FL plasma and urine concentrations in the infants fed human milk and the formula containing 1.0 g 2'-FL/L. Based on these results, Marriage et al. concluded that the absorption of 2'-FL in infants fed 2'-FL-supplemented infant formulas is similar to that in breast fed infants.

Importantly, because the 2'-FL that is the subject of this GRAS Notification is structurally identical to the 2'-FL found in breast milk and the resulting estimated daily intake of 2'-FL from the intended uses falls within the range of 2'-FL intake from breast milk (see Section III.B), there is reasonable certainty that the absorption, distribution, metabolism, and excretion of 2'-FL ingested from the intended uses at the intended use levels will mimic that from breast milk.

C. TOXICOLOGY

The toxicity of 2'-FL has been evaluated in a battery of unpublished and published genotoxicity and subchronic toxicity studies that have been used to support the safety and GRAS status of 2'-FL products (Table 5). Four unpublished studies and one published 21-day neonatal piglet tolerance study conducted by Hanlon et al. (2014) support the safety and GRAS status of the 2'-FL that is the subject of this Notice and are extensively summarized in GRN 571. Additionally, since the Agency's "no questions" letter to the GRN 571 supplement, which summarized the genotoxicity and subchronic toxicity studies published since the filing for GRN 571, a new study that evaluated the genotoxicity and subchronic toxicity of a mixture containing 2'-FL, 3'-FL, LNT, 3'-SL, 6'-SL, all of which are manufactured by Jennewein using carefully controlled fermentation conditions (Parschat et al., 2020). Because the studies published since the filing of GRN 571 have been extensively summarized in previous GRAS Notifications and the supplement to GRN 571 (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 815, 2019), they are all incorporated by reference and briefly summarized below along with the new studies conducted by Parschat et al. (2020). Collectively, all of the published studies show that 2'-FL alone or in the presence of other HMOs is not genotoxic, clastogenic or aneugenic and has a NOAEL of at least 5 g/kg/day in rats. 2'-Fucosyllactose is also well-tolerated up to 0.29 g/kg in neonatal piglets.

Table 5. Toxicity Studies that Support the Use of 2'-Fucosyllactose in Infant Formula

Publication	Manufacturer	Method of Manufacturing	Studies	Conclusions	GRAS Notice
2'-Fucosyllactose					
Coulet et al., 2014	Glycom	Chemical synthesis	Genotoxicity 1. An OECD-compliant bacterial reverse mutation test 2. An OECD-compliant *in vitro* mammalian cell gene mutation assay in mouse lymphoma L5178Y cells Subchronic Toxicity 1. A 14-day oral toxicity range finder study in rats 2. An OECD-compliant 90-day oral toxicity study in rats	Genotoxicity 2'-FL is not mutagenic Subchronic Toxicity NOAEL: 5 g/kg/day	546
Unpublished (summarized in GRN 571)	Jennewein Biotechnology, GmBH	Fermentation	Genotoxicity 1. An OECD-compliant bacterial reverse mutation test 2. An OECD-compliant chromosomal aberration test Subchronic Toxicity 1. A 7-day dietary toxicity study in rats 2. An OECD-compliant 90-day dietary toxicity study in rats	Genotoxicity 2'-FL is not mutagenic, clastogenic, or aneugenic Subchronic Toxicity NOAEL: males=7.6 g/kg/day; females=8.72 g/kg/ day	571
Hanlon et al., 2014	Jennewein Biotechnology, GmBH	Fermentation	21-day in neopiglet tolerance study	NOAEL: males = 0.29 g/kg/ day; females = 0.29 g/kg/ day	571
van Berlo et al., 2018	Friesland Campina Domo	Fermentation	Genotoxicity 1. An OECD-compliant bacterial reverse mutation test 2. An OECD-compliant *in vitro* micronucleus test in cultured human lymphocytes Subchronic Toxicity An OECD-compliant 90-day dietary toxicity study in rats	Genotoxicity 2'-FL is not mutagenic Subchronic Toxicity NOAEL: males=7.25 g/kg/ day; females=7.76 g/kg/ day	735

Table 5. Toxicity Studies that Support the Use of 2'-Fucosyllactose in Infant Formula

Publication	Manufacturer	Method of Manufacturing	Studies	Conclusions	GRAS Notice
Unpublished (Verspeek-Rip et al. (2015, described in GRN 650)	Glycom	Fermentation	Genotoxicity An OECD-compliant bacterial reverse mutation test	2'-FL is not mutagenic	650
Unpublished (Verbaan et al. 2015a, as described in GRN 650)	Glycom	Chemical synthesis	Genotoxicity An OECD-compliant *in vitro* micronucleus test in human peripheral lymphocytes	2'-FL is not clastogenic or aneugenic	650
Unpublished (Verbaan et al. 2015b, as described in GRN 650)	Glycom	Fermentation	Genotoxicity An OECD-compliant *in vitro* micronucleus test in human peripheral lymphocytes	2'-FL is not clastogenic or aneugenic	650
Unpublished (Penard et al., 2015)	Glycom	Fermentation	Subchronic Toxicity An OECD-compliant 90-day dietary toxicity study in rats	NOAEL: 5 g/kg/day	650
Mixtures with 2'-Fucosyllactose					
Phipps et al., 2018 (2'-fucosyllactose and difucosyllactose (DFL)	Glycom A/S	Fermentation	Genotoxicity 1. An OECD-compliant bacterial reverse mutation test 2. An OECD-compliant *in vitro* mammalian micronucleus test in human blood lymphocytes Subchronic Toxicity An OECD-compliant 90-day oral toxicity study in rats	Genotoxicity 2'-FL/DLF is not mutagenic Subchronic Toxicity NOAEL: 5 g/kg/day	815
Parschat et al., 2020 (2'-fucosyllactose, 3-fucosyllactose, lacto-N-tetraose, 3'-sialyllactose, and 6'-sialyllactose)	Jennewein Biotechnology, GmBH	Fermentation	Genotoxicity 1. An OECD-compliant bacterial reverse mutation test 2. An OECD-compliant chromosomal aberration test Subchronic Toxicity 1. A 7-day dietary toxicity study in rats 2. An OECD-compliant 90-day dietary toxicity study in rats	Genotoxicity 2'-FL is not mutagenic, clastogenic, or aneugenic Subchronic Toxicity NOAEL: males = 5.67 g/kg bw/day; females = 6.97 g/kg bw/day	Not cited in a GRN

1. Genotoxicity

a. Studies of 2'-Fucosyllactose as a Single Ingredient

i. Bacterial Reverse Mutation Tests

As summarized in the GRN 571 supplement, the results of two additional OECD-compliant bacterial reverse mutation tests on 2'-FL are now publicly available. van Berlo et al. (2018) evaluated the mutagenic activity of 2'-FL produced by Friesland Campina Domo using fermentation in an OECD 471-compliant bacterial reverse mutation test using the histidine-requiring *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 and the tryptophan-requiring *E. coli* strain WP2 uvrA in the absence and presence of metabolic activation. Five test concentrations of 2'-FL ranging from 62 to 5000 μg/plate were used. In both the absence and presence of metabolic activation, no dose related increase in the mean number of revertant colonies compared to background were reported at concentration up to 5000 μg/plate. The colonies of the negative controls were within the acceptable range and positive controls showed a significant increase in the number of revertant colonies.

Verspeek-Rip et al. (2015, described in GRN 650) evaluated the mutagenic activity of 2'-FL produced by Glycom using fermentation in an OECD 471-compliant bacterial reverse mutation test using *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and an *E. coli* strain WP2uvrA in the presence and absence of metabolic activation. Five concentration of 2'-FL ranging from 52 to 5000 μg/plate were tested. There was no cytotoxicity to any of the strains tested, no significant or dose-related increase in revertant colonies, and no mutagenic effect.

ii. Micronucleus Tests

As summarized in the GRN 571 supplement, the results of three additional OECD-compliant *in vitro* micronucleus tests on 2'-FL are now publicly available. van Berlo et al. (2018) evaluated the clastogenic and aneugenic effects of 2'-FL produced by Friesland Campina Domo using fermentation in an OECD 487-compliant *in vitro* micronucleus test using cultured human lymphocytes. Duplicate cultures of binucleated human lymphocytes, in the absence and presence of a metabolic activation system, were exposed to concentrations of 2'-FL ranging from 3.9 to 2000 μg/mL. Cytotoxicity was determined using the Cytokinesis-Block Proliferation Index. In the first experiment, exposure was for 4 hours with a 20-hour recovery time, and in the second experiment exposure was for 20 hours with no recovery time. Results indicated no statistically significant, dose-related increases in cytotoxicity or in the number of binucleated cells containing micronuclei at any concentration tested in experiment 1 or 2. The number of binucleated cells containing micronuclei were reported to be within the test facility's historical

data range. The authors conclude that 2'-FL is not mutagenic based on the negative results of the *in vitro* micronucleus test.

Verbaan et al. (2015a, as described in GRN 650) evaluated the clastogenic and aneugenic effects of chemically synthesized 2'-FL manufactured by Glycom in an OECD 487-complaint *in vitro* mammalian cell mutation assay using peripheral human lymphocytes. 2'-FL did not increase the number of micronucleated peripheral human lymphocytes at concentrations of up to 2,000 μg/mL in the presence and absence of exogenous metabolic activation (S9).

Verbaan et al. (2015b, as described in GRN 650) evaluated the clastogenic and aneugenic effects of 2'-FL produced by Glycom using fermentation in an OECD 487-complaint *in vitro* mammalian cell mutation assay using peripheral human lymphocytes. In a short-term exposure experiment, lymphocytes were incubated with 2'-FL at concentrations of 512, 1,600, or 2,000 μg/mL for 3 hours in the presence or absence of S9, following which the cells were rinsed and incubated for another 24 hours prior to scoring. In the long-term exposure experiment, cells were treated with 2'-FL at concentrations of 512, 1,600, or 2,000 μg/mL for 24 hours in the absence of S9. At least 1,000 binucleated cells and 1,000 mononucleated were scored for micronuclei under each treatment condition. No significant increase in cytotoxicity or in the number of micronucleated cells in the presence or absence of metabolic activation was reported.

b. Studies of 2'-Fucosyllactose as Part of a Mixture

i. Bacterial Reverse Mutation Tests

Two OECB-complaint bacterial reverse mutation tests have been conducted on mixtures of HMOs containing 2'-FL. As summarized in GRN 650, Phipps et al. (2018) conducted an OECD 471-complaint bacterial reverse mutation test using a mixture of 2'-FL (92.2%) and difucosyllactose (DFL) (9.70%) produced by Glycom using fermentation. In this study *S. typhimurium* strains TA98, TA100, TA1535, and TA1537, and *E. coli* strain WP2 uvrA were exposed to concentrations of 2'-FL/DFL ranging from 5 to 5000 μg/plate in the absence and presence of metabolic activation. Cytotoxicity was evaluated based on revertant colony counts of treated compared to control. The authors reported no dose related increase in the number of revertant colonies in either the presence or absence of metabolic activation at concentrations up to 5000 μg/plate. Mean values for treated cultures, as well as negative and positive controls were within respective historical control data ranges.

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-compliant 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 2.4, 4.7, 14.9, 47.1, 148.8, and 282.6 mg 2'-FL, 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 282.6 mg 2'-FL/plate) in either the plate incorporation or preincubation tests (Table 6). Parschat et al. concluded that the results indicate that the HMO mixture, and the 2'-FL contained therein, was not mutagenic under the conditions tested.

Table 6. Bacterial Reverse Mutation Test Performed with an HMO Mixture Containing 47.1% 2'-Fucosyllactose[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 contained 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.

ii. Micronucleus tests

Two OECD-complaint *in vitro* micronucleus test have been conducted on mixtures of HMOs containing 2'-FL. As summarized in GRN 650, Phipps et al. (2018) performed an OECD 487-compliant *in vitro* mammalian cell micronucleus test using human peripheral blood lymphocytes and a mixture of 2'-FL (92.2%) and difucosyllactose (DFL) (9.70%) produced by fermentation (Glycom). The lymphocytes were exposed to concentrations of the 2'-FL/DFL mixture ranging from 500 to 2000 μg/plate in the presence and absence of metabolic activation for 3 hours of in the absence of metabolic activation for 20 hours. No treatment related changes in clastogenicity or aneugenicity at concentrations up to 2000 μg/plate in the presence or absence of metabolic activation were reported. The mean values for exposed cultures, as well as, negative and positive controls were within respective historical control data ranges.

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 408-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 3.5, 7.1, 14.1, and 28.3 mg 2'-FL/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 (Table 10), both validating the tests. In contrast, no chromosomal damage was observed with the HMO mixture at any concentration or under any condition tested (Table 7). Thus, the HMO

mixture was not genotoxic under the tested conditions at concentrations up to 60 mg/mL (28.3 mg/mL 2'-FL).

Table 7. *In vitro* Micronucleus Test in Human Peripheral Blood Lymphocytes Exposed to an HMO Mixture Containing 47.1% 2'-Fucosyllactose[b]

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

Values are means (n = 2).
CBPI = Cytokinesis block proliferation index; RI = Replicative Index.
[a]Significantly different from negative control ($p \leq 0.05$).
[b]The HMO mixture also contained 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.

2. Toxicity Studies on 2'-FL as a Single Ingredient

a. Studies of 2'-Fucosyllactose as a Single Ingredient

Two 90-day 2'-FL toxicity studies in rats have been conducted since the filing of GRN 571 (van Berlo et al., 2018; Penard et al. 2015 cited in GRN 650). As summarized in GRN 735, van Berlo et al. (2018) administered 2'-FL manufactured by Friesland Campina Domo using fermentation in the diet at concentrations of 0, 3, 6, and 10% to male and female Wistar Han IGS rats (Crl:WI(Han); 10/sex/group) for 13 weeks in an OECD 480-compliant 90-day dietary toxicity study. The diets were analyzed for stability, homogeneity, and concentration of 2'-FL throughout the study. Feed intake was reported to decrease with increasing age of the rats; therefore, the intake of 2'-FL per kilogram body weight decreased in all groups during the study. The overall mean 2'-FL intakes were 2.17, 4.27, and 7.25 g/kg/day for males and 2.45, 5.22, and 7.76 g/kg/day for females. Results following dietary intake of 2'-FL for 13 weeks produced no exposure-related changes in mortality or clinical signs in any of the treated groups. Results of the functional observational battery and motor activity assessment did not indicate any neurotoxic potential for 2'-FL. No significant differences were noted between controls and treated groups. No changes in feed consumption in male rats was reported; however, feed consumption in the high-dose females was significantly decreased. Hematology results indicated a significant increase in thrombocytes in the high-dose females; however, this finding was determined by the authors to be a chance finding because the difference from controls was only slight and occurred in one sex only. No other hematological or clinical chemistry changes were noted in the treated groups. Results of renal concentration tests showed a significantly decreased specific gravity in female in the high dose group. The authors attributed the change to a higher urinary excretion volume and the change was not considered toxicologically significant. Relative liver weight was significantly increased in the high dose males and absolute and relative weights of the filled and empty cecum were significantly increased in the mid- and high-dose group in male and female rats. In addition, the absolute weight of the filled cecum was significantly increased. No significant macroscopic or microscopic changes related to treatment were reported in any of the treatment groups. van Berlo et al. (2018) concluded that ingestion of 2'-FL for 13 weeks produced no treatment-related changes in male and female rats and reported a NOAEL at the highest concentrations tested, corresponding to ≥7.25 g/kg/day in male rats and ≥7.76 g/kg/day in female rats.

As summarized on pg. 31 of GRN 650, Penard et al. (2015) evaluated the toxicity of a 2'-FL manufactured by fermentation (Glycom) in an adapted 90-day oral toxicity, which involved 7-day-old neonatal Wistar [Crl:WI(Han)] rats. Either 2,000, 4,000, or 5,000 mg 2'-FL/kg body weight/day was administered to 7-day-old neonatal Wistar [Crl:WI(Han)] rats via gavage for 90-days. A reference group was also included that received fructooligosaccharides (FOS) at a dose

of 5,000 mg/kg body weight/day. Separate recovery groups consisting of 5 males and 5 females administered the control, 2'-FL, or FOS for 90 days were terminated after a 28-day recovery period. Individual dams with reconstituted litters of at least five male and five female pups were housed in plastic cages until weaning on post-natal day (PND) 21. All pups in each reconstituted litter were treated at the same dose level as the dams (starting on PND 7). On PND 21, pups were weaned and placed in plastic cages according to sex and dose group such that a total of 5 pups of the same sex and dose group were housed per cage. A standard diet and water were provided *ad libitum*. Animals were observed twice daily for mortality and morbidity, and clinical observations were performed daily. A detailed clinical examination was performed weekly. Body weights were assessed at time of randomization, prior to dosing, twice weekly during the first 8 weeks of the administration period, and then once weekly thereafter. Feed intake also was measured twice weekly after weaning and for the first 6 weeks post-weaning, and then once weekly thereafter. Ophthalmological examinations were performed on all animals from the control, high-dose 2'-FL, and FOS groups during the last week of administration. Fasting blood and urine samples were collected from all animals of all groups for clinical pathology analysis (i.e., hematology, coagulation, clinical chemistry, and urinalysis) at the end of the administration period. Clinical pathology also was performed for all animals from all groups at the end of the recovery period. Complete necropsy was performed and selected organs were removed and weighed for all animals at the end of the treatment period or at the end of the 4-week recovery period. Histopathological examinations of all organs and tissues were performed for all animals in the control, high-dose 2'-FL, and FOS groups at the end of the administration period. Kidneys from all females in the low- and mid-dose groups and in all recovery groups also were microscopically examined.

No test article-related mortalities occurred during the study. The majority of animals receiving the reference compound presented with liquid feces, which was also observed in mid- and high- dose animals receiving 2'-FL. Mid- and high-dose animals receiving 2'-FL also had soiled urogenital regions. Hypersalivation, abnormal foraging and/or pedaling were observed in animals receiving the reference compound and also in the mid- and high-dose groups receiving 2'-FL from day 35 onward; however, these clinical signs did not persist during the recovery period. No test article- related ophthalmological findings were observed. No remarkable effects in body weight, body weight gain, or feed consumption were observed. No toxicologically relevant effects in tibia length, reflex and physical development, time to sexual maturation, learning capacity, memory, motor activity (as evaluated in the Morris water maze), exploratory behavior, or general movement (as evaluated in the open-field test) were observed at any dose level.

Minor differences in certain hematological parameters were deemed to be of no toxicological significance. Triglyceride concentrations were decreased in mid- and high-dose

males receiving 2'-FL compared with the water control group and the FOS reference group. Cholesterol concentrations also were decreased in low-, mid-, and high-dose males receiving 2'-FL and in females receiving mid- and high-dose 2'-FL as compared to the water control group. Individual urea concentrations also were noted to be high for a few animals receiving high-dose 2'-FL. However, it was noted that overall, these changes in serum chemistry parameters were low in magnitude and/or within the normal historical control data range for this laboratory and strain of rat. Additionally, the differences in serum parameters were not observed following the recovery period. Thus, it was concluded that no adverse effect of treatment was observed in serum biochemical parameters.

No test article-related differences in urinalysis parameters were observed between treatment groups and the water control or reference compound. No treatment-related differences in organ weights or macroscopic observations were observed between rats receiving 2'-FL and the control and reference groups. No evidence of treatment-related effects in histological observations was observed in animals receiving 2'-FL compared to control and reference groups.

Penard et al. (2015) also reported no treatment-related changes to support evidence for the lack of toxic effects of 2'-FL in a 90-day oral toxicity study and the authors concluded that the NOAEL was 5 g/kg/day, the highest dose tested.

b. Studies of 2'-Fucosyllactose as Part of a Mixture

A seven-day feeding toxicity study and two OECD-complaint 90-day toxicity studies in rats have been conducted on mixtures of HMOs containing 2'-FL.

i. Seven-day Dietary Toxicity Study

In a seven-day pilot feeding toxicity study, female CD/Crl:CD rats (Charles River Laboratories, Sulzfeld, Germany) received either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Jennewein (n=5/group). All animals were individually housed. The HMO mixture contained 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL and 5.1% dry weight other carbohydrates, all of which were manufactured by fermentation. Thus, the overall dietary exposure to 2'-FL was 4.71 % of the diet. Both diets were provided ad libitum. Animals were observed daily for viability, behavioral changes, and reactions to treatment or illness. Cage-side observations included skin and fur, eyes, mucous membranes, respiratory and circulatory systems, somatomotor activity, behavior patterns, and feces output and consistency. Body weight was recorded at the time of group allocation, on the 1st day of treatment and daily thereafter at the same time each day. Feed consumption was recorded daily and feed intake per rat (g/rat/day) was calculated subtracting the

total amount of feed left from the total amount of feed given and dividing the difference by the number of days and body weight of the rat. Drinking water consumption was monitored daily by visual inspection. Intake of the test article was calculated on a daily and weekly basis throughout the experimental period based on the concentration in the diet, individual feed intake and body weight of each rat. No mortalities occurred during the study. No HMO-related differences in behavior, appearance and consistency of the feces, body weight, body weight gain, or feed consumption were observed. Thus, the dose of 10% HMO mixture in diet (47.1% 2'-FL by dry weight, providing 2'-FL as 4.7% of total diet) was chosen for the subsequent 13-week dietary toxicity study in rats.

ii. Thirteen-Week Toxicity Studies

As summarized in the GRN 650, Phipps et al. (2018) conducted an OECD 408-compliant 90-day repeated dose oral toxicity study with 2'-FL/DFL, manufactured by Glycom using fermentation, in male and female Sprague-Dawley rats. An 8:1 ratio mixture of 2'-FL and difucosyllactose (DFL) was administered via oral gavage to neonatal rats daily at 0, 1000, 3000, and 5000 mg/kg bw/day of 2'-FL/DFL for 90 days followed by a 28-day recovery period. No mortality or exposure-related clinical signs were observed. Mean body weight and food consumption did not differ significantly between treatment groups and vehicle. Furthermore, the authors reported no treatment-related adverse effects with a dose-response relationship were observed for development and maturation, behavioral endpoints, clinical pathology, organ weights, or histopathology. Phipps et al. (2018) concluded that the NOAEL for the 2'-FL/DFL mixture was 5,000 mg/kg bw/day, the highest dose tested.

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 2'-FL was 4.7% 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 and recorded.

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 adrenal glands, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, uterus (including cervix), and prostate and seminal vesicles, 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 bw/day for male rats and 6.26 to 7.91 g/kg bw/day for the female rats. This resulted in a mean intake of 2'-FL of 2.36 to 3.24 g/kg bw/day in males and 2.95 to 3.73 g/kg bw/day in females.

Prior to and over the course of four weeks of the 13-week study, one male animal in the control group (standard diet) gained weight at a slower rate compared to the other control animals. From six days prior to the study to day 29, the male gained weight at 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, enlarge and thickened mesenteric lymph node, and enlarged spleen were noted at necropsy. Hematology revealed an increased number of leucocytes (9-fold) caused by increased numbers of neutrophilic granulocytes (26-fold), lymphocytes (4-fold), monocytes (19-fold), eosinophilic granulocytes (43-fold), large unstained cells (15-fold) and basophilic granulocytes (24-fold) compared to the mean values for the group. Clinical chemistry revealed increased plasma level of bilirubin (3-fold) and increased enzyme activities of alanine aminotransferase (8-fold), alkaline phosphatase (2-fold), aspartate aminotransferase (12-fold) and lactate dehydrogenase (3-fold). Due to the magnitude of the hematological and clinical chemistry changes, the effects were deemed spontaneous and incidental and the animal was excluded from all analyses.

The HMO mixture had no effect on feed consumption, water consumption, body weight, or body weight gain in either males or females. Except for the one rat that was euthanized moribund and excluded from all analyses, no other mortalities were observed during the study, and no changes in behavior, external appearance, or consistency of feces were recorded in either group. No ophthalmological or auditory changes or effects on body posture, movement, or coordination were observed. Neurological screening revealed no test article-related effects. Although a significant ($p \leq 0.05$) increase in body temperature was reported in female rats in the HMO mix group (38.5 ± 0.3 °C) compared to the control group (38.1 ± 0.4 °C), the decrease was small (approximately 1%), occurred only in females, and was not associated with any other clinical observations. Additionally, male rats in the HMO mix group showed a significant decrease ($p \leq 0.05$) in spontaneous motility (number of movements recorded over a period of 12 min), with a mean value of 96.3 ± 50.3 compared to 167.7 ± 73.9 in the control male rats. Further inspection of the individual rat data revealed that the decrease was due to two males in the control group having spontaneous motilities higher than the upper boundary of the historical range for the laboratory (224 and 299 movements/12 min vs an upper boundary of 217 movements/12 min; laboratory historical control mean of 77.7 movements/12 min). Thus, the increase in body temperature and decrease in spontaneous mobility were deemed to be incidental and not related to the HMO mixture.

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

For the neutrophils, the mean cell counts were generally low relative to the historical control range for the laboratory ($0.4\text{-}12.81 \times 10^3$ cells/μl) in both the control and HMO mix groups. Additionally, although the absolute number in one female receiving the HMO mix fell below the lower boundary of the historical control range (0.33×10^3 cells/μl), all neutrophil

counts in the remaining males and females fell within the historical range. Thus, the statistically significant reduction in the absolute number of neutrophilic granulocytes observed in female rats administered HMO mix was deemed to be unrelated to test article administration.

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

Table 8. 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 9. Significant Differences in Mean Brain and Kidney Weights

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 10. 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 47.1% 2'-FL by dry weight) at 10% of diet for 13 weeks. Based on feed intake data, the NOAEL for this study was 5.67 g/kg bw/day for male rats and 6.97 g/kg bw/day for the female rats. This resulted in a mean intake of 2'-FL of 2.67 g/kg bw/day in males and 3.28 g/kg bw/day in females.

D. TOLERANCE STUDY IN NEONATAL PIGLETS

1. Introduction

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

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. All piglets were housed individually in single-sized stainless-steel cages with plastic coated flooring. 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 study design was as follows (Table 11):

Table 11. Experimental Design

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

[a] Group 1 received ProNurse® only.

[b] Groups 2 and 3 received ProNurse® with Oligosaccharide Blend

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

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

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

Clinical Pathology: Hematology, coagulation, clinical chemistry and urinalysis sample collection were performed as detailed in Table 12.

Table 12. Clinical Pathology Sample Collection Plan

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

X = Sample was collected; NA = Not applicable

[a]Animals were bled at each time point with the exception of collections impacted by unscheduled deaths.

[b]Day 22 at necropsy only.

[c]Additional blood samples were obtained due to sample quality or volume as permissible. Suitable methods were used for unscheduled collections and/or redraws.

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

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

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

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

Gross examination: Animals surviving until scheduled euthanasia were euthanized by an intravenous euthanasia solution administration under sedation followed by a Testing Facility SOP approved method to ensure death. When possible, the animals were euthanized rotating across dose groups such that similar numbers of animals from each group, including controls were necropsied throughout the day. If an animal was in overt pain/distress or appeared moribund and was beyond the point where recovery appears reasonable, the animal was euthanized for humane reasons in accordance with the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia and with the procedures outlined in the protocol. All animals were subjected to a necropsy examination, which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. The animals were examined thoroughly for external abnormalities including palpable masses.

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

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

3. Results

Dose Formulation Analyses: Homogeneity and concentration analyses results of the 5.75 and 8.0 g/L formulations prepared on Day 1 and Day 20 ranged from 93.3% to 94.1%, respectively, of the targeted dose levels and confirmed that formulations were homogenous and animals received the appropriate concentrations (Table 13).

Table 13. 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 13). 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 15).

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

Table 15. Piglets Receiving Antibiotic (LA200 (oxytetracycline injectable solution)) During the Study

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

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

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

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

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

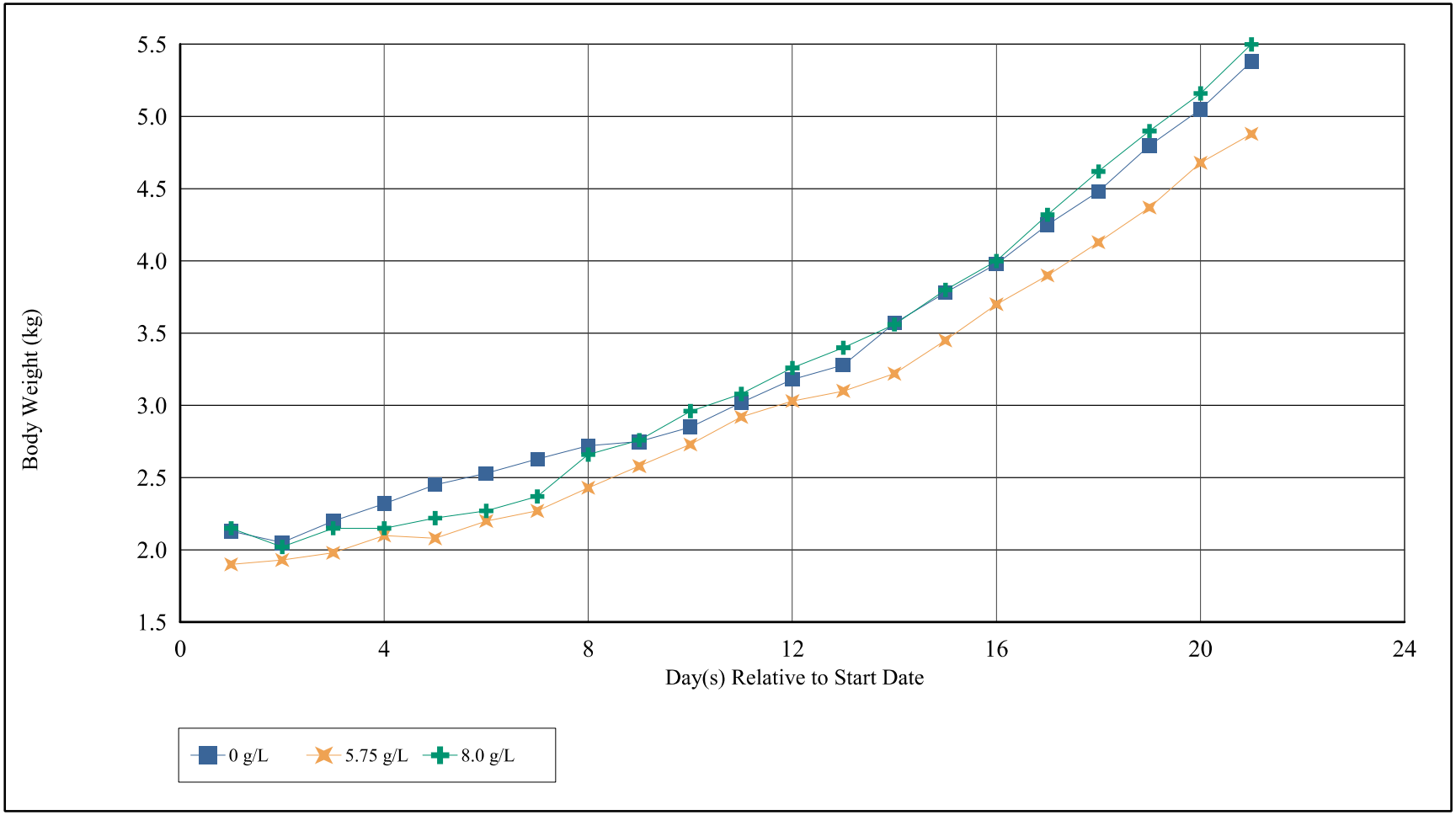


Figure 2a. Mean Body Weight Values (Male)

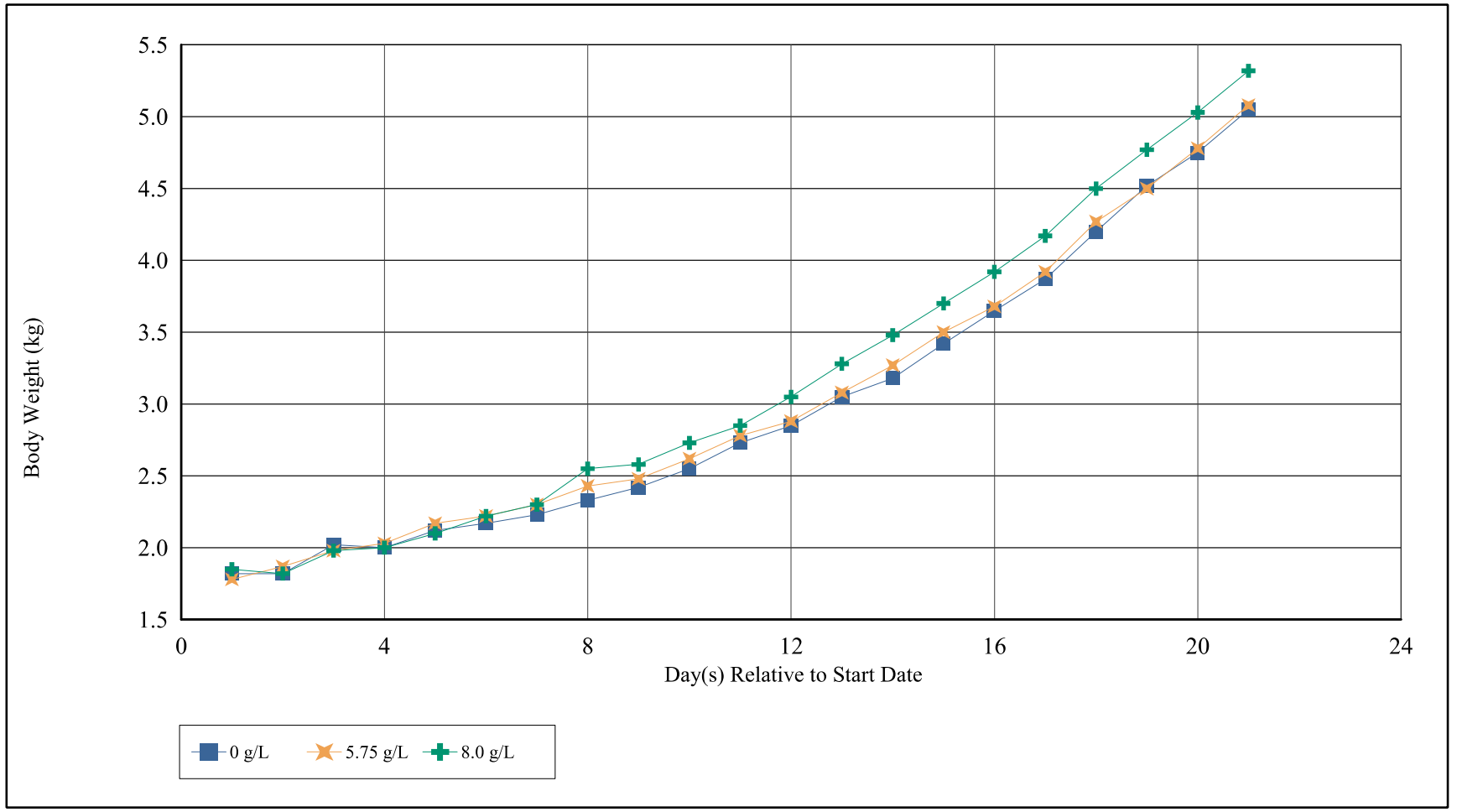


Figure 2b. Mean Body Weight Values (Female)

Table 16. 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						

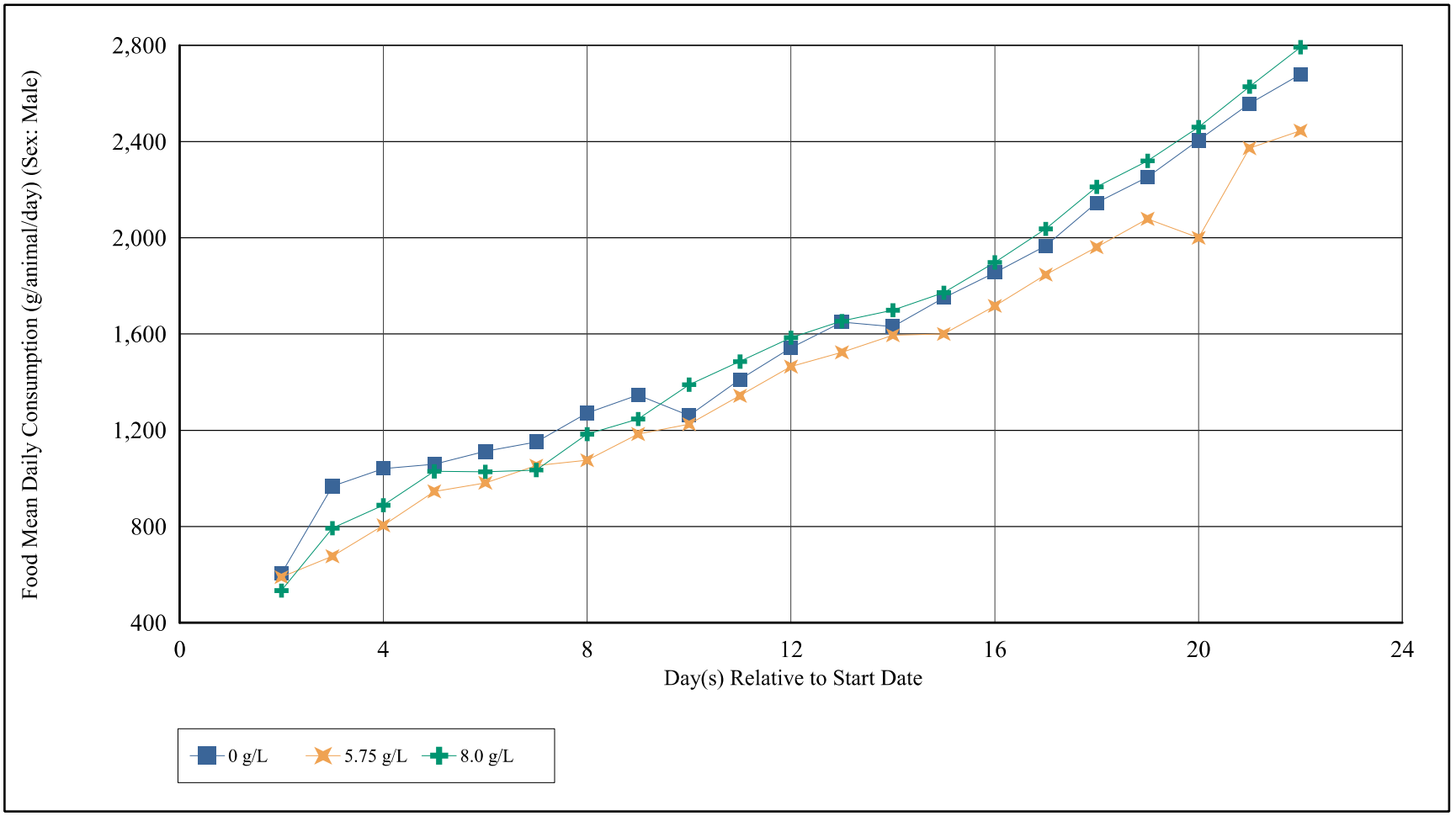


Figure 3a. Mean Feed Consumption Values (Male)

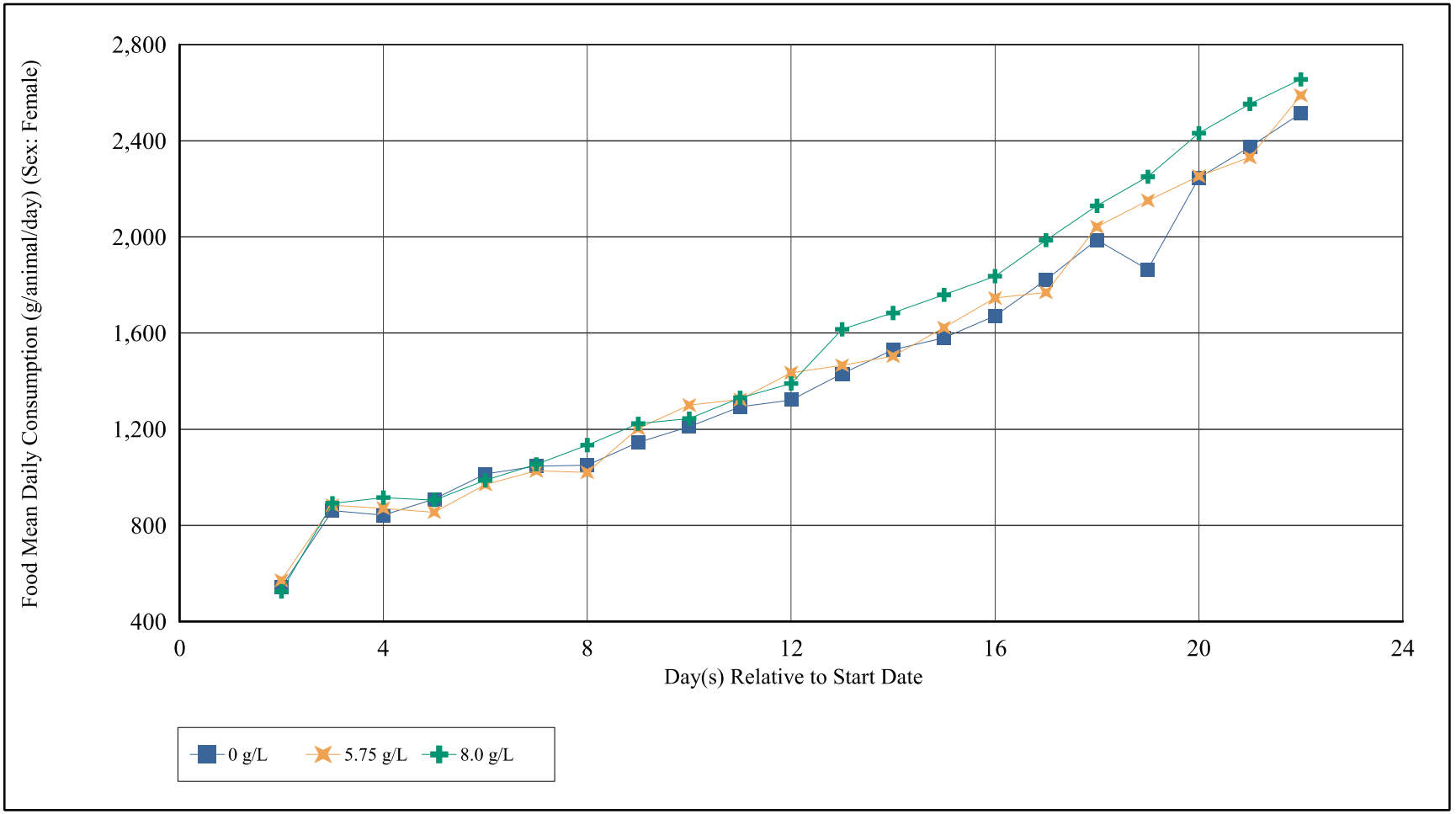


Figure 3b. Mean Feed Consumption Values (Female)

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

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

Clinical Pathology:

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

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

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

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

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

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

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

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

Table 19. Hematology

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 19. Hematology

		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 20. Coagulation Parameters

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

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

Table 21. 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]

Table 21. 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**
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)
	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							

Table 21. 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**
[g] – ANOVA & Dunnett [g1] – Kruskal-Wallis & Dunn [I] – n = Inappropriate for statistics a = p <0.01							

Table 22. 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 23). No microscopic correlates were observed to account for the increased cecum weights.

Table 23. Summary of Large Intestinal Weight Data – Scheduled/Terminal Euthanasia (Day 22)

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

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

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

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

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

Table 24. Absolute and Relative Organ Weight Values							
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 24. Absolute and Relative Organ Weight Values							
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 24. Absolute and Relative Organ Weight Values

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 24. Absolute and Relative Organ Weight Values							
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 24. Absolute and Relative Organ Weight Values

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). These 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 mortalities were identified. The clinical pathology values and macroscopic and microscopic findings at necropsy did not reveal a relationship to treatment with the Oligosaccharide Blend at the concentrations evaluated. Organ weight changes were limited to increased cecum weights in males and females at ≥5.75 g/L, increased colon weights in males at ≥5.75 g/L, and decreased rectum weights in males and females at 8.0 g/L, but these changes were not considered adverse as there were no microscopic correlates. Additionally, studies have shown that nondigestible oligosaccharides (such as inulin and galactooligosaccharides) increase microbial fermentation and result in the production of osmotically active by-products, for example, short-chain fatty acids, which can cause soft stools and colon and cecal weight increase/enlargement (Aufreiter et al. 2011; Kruger et al. 2017). No adverse findings in gross or histopathology were noted.

E. CLINICAL STUDIES

Since the filing of GRN 571, five clinical studies have been published that evaluated safety and tolerance of 2'-FL-supplemented infant formulas and foods, Marriage et al. (2015), Goehring et al. (2016), Elison et al. (2016), Storm et al. (2019), and Kajzer et al. (2016). Marriage et al. (2015), Elison et al. (2016), and Goehring et al. (2016) were comprehensively summarized in previous 2'-FL GRAS Notices (GRN 650, 2016; GRN 735, 2018). Storm et al. (2019) and Katjzer et al (2016) were comprehensively summarized in the GRN 571 supplement. Therefore, the summaries provided in the previous GRNs and the GRN 571 supplement are incorporated by reference, and the studies are briefly summarized below.

1. Studies in Infants

As summarized on p. 37 of GRN 650, Marriage et al. (2015) conducted a prospective, randomized, placebo-controlled, double-blind study to examine growth and tolerance of infant formulas having a caloric density approximating human milk supplemented with chemically synthesized 2'-FL and study the uptake of the 2'-FL in infants. Infants were enrolled within Day of Life (DOL) 5 and consumed either a standard, milk-based, commercially-available infant formula containing 2.4 g galactooligosaccharides (GOS) (n=101), a standard formula supplemented with 0.2 g 2'-FL/L and 2.2 g GOS/L (104), a standard infant formula supplemented with 1.0 g 2'-FL/L and 1.4 g GOS/L (109), or breast milk from their mothers (n=106) for 4 months. All formulas had a caloric density of 64.3 kcal/dL, which is comparable to human milk. Growth was measured using weight, length and head circumference. Tolerance was measured by average stool consistency, number of stools per day, and percent of feedings associated with spit- up or vomit. 2'-Fucosyllactose absorption was measured by quantitating the levels of 2'-FL in infant plasma and urine in a subset of infants at Day of Life 42 and 119 and from the human milk of the breast-feeding mothers at Day of Life 42.

Three hundred thirty-eight infants completed the study, 304 of whom completed the study on the assigned feeding or human milk. The number of premature discontinuations on the study formulas was not different among the formula-fed groups. There were no significant differences among any of the groups in weight, length, or head circumference during the study. All formulas were well-tolerated and there were no significant differences in the overall percentage of infants with adverse events or serious adverse events between the infants receiving the formulas containing 2'-FL and GOS and the standard formula. The formulas also resulted in comparable stool consistency, number of stools/day, and percent feedings associated with spitting up or vomit. No 2'-FL was detected in the plasma of infants fed the standard milk-based commercial formula containing GOS, whereas 2'-FL was detected in the plasma and urine of infants provided the 2'-F-containing formula or consuming human milk, with the greatest mean

2'-FL plasma and urine concentrations in the infants fed human milk and the formula containing 1.0 g 2'-FL/L. Based on these results, Marriage et al. concluded that the feeding of infant formula with a caloric density similar to that of human milk resulted in comparable growth rates to that of human milk-fed infants, formulas supplemented with 2'-FL were well-tolerated, and the absorption of 2'-FL from 2'-FL supplemented formulas is similar to that from breast milk.

As summarized on pg. 62 of GRN 735, Goehring et al. (2016) investigated the effects of feeding formula supplemented with 2'-FL on biomarkers of immune function in a sub-study of the clinical trial conducted by Marriage et al. (2015). Of the 424 infants enrolled in the original clinical trial, 315 infants participated in the sub-study. Non-fasting venous blood was collected at six weeks of age and analyzed for respiratory syncytial virus (RSV) load, plasma cytokines, peripheral blood mononuclear cells (PBMC) phenotyping, and quantitation of ex vivo PBMC phytohemagglutinin (PHA)-stimulated proliferation and cytokine production and respiratory syncytial virus (RSV)-simulated cytokine secretion.

There was no difference in RSV loads among any of the groups. Breastfed infants and infants fed either of the formulas containing 2'-FL had comparable levels of plasma inflammatory cytokines, which were 29-83% lower than the levels found in the infants fed the standard formula containing only GOS. Peripheral blood mononuclear cells (PBMC) phenotyping was not significantly different between the infants ingesting the control and 2'-FL-supplemented formulas. *Ex vivo* PHA- and RSV-stimulated proliferation and proinflammatory cytokine secretion of PMBCs were also similar among the infants consuming the different formulas. Thus, Goehring et al. concluded that infants fed formula supplemented with synthesized 2'-FL exhibited lower plasma and ex vivo inflammatory cytokine profiles, and were similar to those of the breastfed reference group.

As summarized on page 38 of GRN 650, Puccio et al. (2017) conducted a double-blind, randomized, controlled clinical trial in 175 healthy, full term infants age 0 to 14 days. The infants were randomly assigned to groups receiving formula containing a combination of 2'-FL (1 – 1.2 g/L) and lacto-*N*-*neo*tetraose (LNnT) (0.5 – 0.6 g/L) (n = 88) or formula that did not contain either oligosaccharide (n = 86) for up to 6 months. No significant differences in weight gain between the test and control groups were reported and the mean weight, length, head circumference, and body mass index (BMI) for all infants through age 4 months were comparable with the WHO standard growth curves. There were also no changes in stool endpoints or microbiota composition in infants receiving the 2'-FL/LNnT supplemented formula. Infants treated with 2'-FL and LNnT had significantly lower incidences of bronchitis, as well as significantly lower antibiotic use compared to infants in the control group according to the authors. There were no other significant differences noted between treated and control groups.

As summarized on page 21 of the GRN 571 supplement, Kajzer et al. (2016) conducted a prospective, randomized, multi-center, double blinded, controlled, tolerance trial in healthy term infants 0 to 8 days old to assess the gastrointestinal tolerance of a formula supplemented with 0.2 g 2'-FL/L and 2.0 g short-chain fructooligosaccharide (scFOS)/L compared to formula without 2'-FL or GOS, or breast milk over 35 days. The authors reported no significant differences in stool consistency, formula intake, anthropometric measures, or percent feedings with spit-up or vomit associated with feedings across the three groups at 35 days of age. Kajzer et al. concluded that the formula supplemented with up to 0.2 g/L 2'-FL and 0.2 g/L scFOS was safe and well tolerated in infants.

As summarized on page 21 of the GRN 571 supplement, Storm et al. (2019) conducted a randomized, placebo-controlled, double-blind study in health infants 14 days of age to assess the tolerance of a new partially-hydrolyzed whey protein infant formula containing *Bifidobacterium animalis* ssp *lactis* Bb12 with and without 0.25 g/L 2'-FL. After 6 weeks of feeding the different formulas, the primary outcome of tolerance was assessed using the Infant Gastrointestinal Symptom Questionnaire (IGSQ). Stooling, vomiting, spit-up, crying, and fussing were compared between the groups. Adverse events were also recorded.

IGSQ scores were similar at baseline and after 6 weeks of exposure. Stool frequency and consistency were similar among the test and control groups throughout the study. More stools were reported to be difficult to pass in the control subjects compared to the test group ($p = 0.04$); however, the number of infants with difficulty passing stools did not differ between groups. Crying, fussing duration, and vomiting frequency were similar between the two groups. Average formula intake, body weight, and body lengths did not differ between the control and test groups. There were more subjects with spit-up noted as frequent in the test group compared to controls. More subjects reported infections in the control group compared to the tested group. Storm et al. concluded that the partially hydrolyzed formula with 2'-FL and *B. animalis* ssp *lactis* Bb12 was well tolerated.

2. Studies in Adults

As summarized on page 61 of GRN 735, Elison et al. (2016) conducted a double-blind, parallel, randomized, placebo-controlled study in 100 healthy adults (19 – 57 years old) to determine the tolerance of 2'-FL and/or LNnT. The study participants (51 males and 49 females) received 5, 10 or 20 g of either 2'-FL, LNnT or 2'-FL with LNnT (2:1 mass ratio) or 2 g of glucose as the placebo each day at breakfast. To evaluated tolerance, the participants completed a self-administered gastrointestinal symptom rating scale (GSRS) form reporting on abdominal pain, indigestion, reflux, diarrhea, and constipation which were ranked from 1 (no discomfort) to 7 (very severe discomfort), bowel movement frequency, and stool consistency at screening at

entry to the study, and at the end of the intervention period. Blood samples were collected at screening and at the end of the intervention and were analyzed for hemoglobin, erythrocytes, hematocrit, leucocytes, thrombocytes, creatinine, sodium, potassium, alanine aminotransferase, alkaline phosphatases, coagulation factor II, VII and X, bilirubin, albumin, C-reactive protein and glucose as well as HbA1c, apoA1, apoB, transferrin, progesterone, cortisol, estradiol, interleukin-10, interleukin-6, tumor necrosis factor-α, blood urea nitrogen, iron, TAG, HDL-cholesterol, LDL-cholesterol, total free fatty acids, insulin, lysozyme, testosterone and glucagon. Fecal samples were collected prior to study entry and at the end of the intervention and analyzed for calprotectin, secretory IgA, and short-chain fatty acid levels as well as fecal microbiota composition.

No participants dropped from the study and forty-four participants reported a total of fifty-six mild adverse events, which were a combination of symptoms including flatulence, bloating, and constipation. The participants taking the highest doses of 2'-FL and LNnT reported the most adverse events. Flatulence was the most commonly reported adverse event followed by stomach pain, diarrhea or loose stool, and borborygmus. The reports of bloating and gas were significantly higher in the 20 g 2'-FL and LNnT groups. The 20 g 2'-FL group also reported increased rumbling. The mean GSRS scores were low (mean score of < 3 which is mild discomfort or below) and those subjects receiving the highest dosages did not have statistically significant changes in their GSRS. Changes in the average number of daily bowel movements were small, though statistically higher in the 20 g 2'-FL, 20 g LNnT, and 5 g LNnT groups (increase of 0.3 movements per day compared to baseline) but the authors deemed this as clinically irrelevant. The authors stated that participants receiving 20 g 2'- FL reported softer stools compared to baseline. Because many of the participant reported events were common gastrointestinal symptoms, Elison et al. stated that it was difficult to determine if they were due to treatment or normal variation and increased participant awareness of gastrointestinal symptoms during the study period. Elison et al. also reported that all measured clinical chemistry and hematology parameters remained within normal ranges. Elison et al. concluded that 2'-FL is safe and well tolerated at concentrations up to 20 g 2'-FL per day for 14 days in healthy adults.

F. ALLERGENICITY

The potential allergenicity of the subject of this Notice is summarized in the GRN 571 supplement, which received a 'no questions" letter from FDA on November 9, 2019, and therefore incorporated by reference (pg. 21). Briefly, no allergenic material per Regulation (EU) No. 1169/2011 are used in the production of Jennewein 2'-FL other than lactose from cow's milk, the fermentation process does not use antibiotics or inhibitors, the manufacturing process does not use organic solvents, and batch data demonstrate that the product is consistently devoid of protein, bacteria, bacterial endotoxins, residual DNA, antibiotics, and chemical sensitizers

including metals, or that they are well below the levels of concern. Therefore, the potential allergenicity of Jennewein 2'-FL is expected to be extremely low.

G. REGULATORY APPROVALS AROUND THE WORLD

In the United States, 2'-FL, including Jennewein 2'-FL, is GRAS for use in infant formulas at levels up to 2.4 g/L and selected conventional foods and beverages at levels ranging from 0.28 to 1.2 g/serving (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019). Eight GRAS Notifications have been filed with FDA. Seven of the eight Notifications received "no questions" letters and the Notifier of the remaining GRAS Notification ceased FDA's evaluation due to major deficiencies. A mixture of 2'-FL and difucosyllactose is also GRAS for use in infant formula, toddler formula, drinks for young children and selected conventional foods and beverages (GRN 815, 2019).

Outside the United States, 2'-Fucosyllactose is a Novel Food in the European Union, Australia, and New Zealand and approved for use in infant formula and selected foods alone or in combination with lacto-*N*-*neo*tetraose at levels up to 1.2 g/L and 200 g/kg, respectively. It is also a Novel Food in Canada and authorized in Malaysia, Taiwan, Singapore, Israel, and the Philippines.

VII. SUPPORTING DATA AND INFORMATION

A. REFERENCES

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

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B. EXPERT PANEL STATEMENT

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of 2'-Fucosyllactose (2'-FL) 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 2'-FL 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 2'-FL 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 % 2'-FL dry weight.

    a. 2'-Fucosyllactose is a neutral, fucosylated oligosaccharide in human milk.

    b. The 2'-FL that is the subject of this GRAS Notice is structurally identical to the 2'-FL present in human breast milk.

    c. The subject of this GRAS Notice is also the subject of GRN 571 and the supplement to GRN 571, both of which received "no questions" letters from the United States Food and Drug Administration.

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

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

f. 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).

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

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

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

3. Published studies show that the amount of 2'-FL in breast milk ranges from 0 to 13.8 g/L, with means and medians ranging from 0.01 to 4.6 and 0.01 to 5.2 g/L, respectively.

4. Additional genotoxicology and subchronic toxicology studies published and/or conducted since the filing of GRN 571 show that 2'-FL is not genotoxic and has a No Observed Adverse Effect Level (NOAEL) of 5 g/kg/day in rats and 0.29 g/kg/day in neonatal piglets.

5. The addition of 3.64 g/L 2'-FL in infant formula will result in mean and 90th percentile intakes of 3.3 g/day (0.52 mg/kg/day) and 4.6 g/day (0.56 g/kg bw/day), respectively, for infants 0 to 5 months-old and 0.29 g/day (0.32 mg/kg/day) and 4.0 g/day (0.36 g/kg bw/day) for infants 6 to 11 months-old.

6. The safety of exposure to Jennewein's 2'-FL ingredient at its intended use level is supported by:

a. Published studies that quantitate the levels of 2'-FL in human milk;

e. Analytical data demonstrating that the 2'-FL produced by Jennewein is structurally identical to 2'-FL from human milk;

b. The published and unpublished genotoxicology and subchronic toxicology studies showing that 2'-FL is not genotoxic and has a No Observed Adverse Effect Level (NOAEL) of 5 g/kg/day in rats.

c. Corroborative unpublished and published tolerance studies in neonatal piglets showing that up to 3.92 g/L of Jennewein-manufactured 2'-FL alone and in the presence of other HMOs was well-tolerated and supported normal growth in neonatal piglets.

Therefore, 2'-FL is safe and GRAS at the proposed level of addition to the intended infant formula. 2'-Fucosyllactose 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: 

Date: March 19, 2020

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

Signature: 

Date: March 19, 2020

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

Signature: 

Date: March 19, 2020

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel

Signature: 

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
000924	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

2'-Fucosyllactose (2'-FL)

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

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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

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

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

☒ a) GRAS Notice No. GRN 571

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRNs 546, 650, 735, 749, 815

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

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

Jennewein intends to use 2'-FL as an ingredient in cow's milk-based, non-exempt term infant formula.

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

(Check one)

☐ Yes ☒ No

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

(Check one)

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

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

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

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

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

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

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

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

Other Information

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

☐ Yes ☒ No

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

☐ Yes ☐ No

1. The undersigned is informing FDA that Jennewein Biotechnologie GmbH

(name of notifier)

has concluded that the intended use*(s)* of 2'-Fucosyllactose (2'-FL)

(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 12:40:51 -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 2-FL GRAS Final to FDA.pdf	Submission
	References	Submission

From:	Dietrich Conze
To:	Hice, Stephanie
Cc:	Claire Kruger; Kathy Brailer
Subject:	Re: GRN 000924 - Questions for Notifier
Date:	Wednesday, August 19, 2020 1:24:20 PM
Attachments:	Response to FDA Questions on GRN924 8-19-20.docx

Hi Stephanie.
Our responses to your questions are attached. If you have any additional questions, please let me know.
Regards.
Dietz

Dietrich Conze, PhD
Managing Partner
Spherix Consulting Group
11821 Parklawn Drive, Suite 310
Rockville, MD 20852

Tel: 240-367-6089
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dconze@spherixgroup.com

> On Jul 20, 2020, at 3:36 PM, Hice, Stephanie <Stephanie.Hice@fda.hhs.gov> wrote:
>
> Dear Dr. Conze,
>
> During our review of GRAS Notice No. 000924, we noted further questions that need to be addressed and are attached to this email.
>
> We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your responses.
>
> If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.
>
> Sincerely,
>
> Stephanie Hice
>
> **Stephanie Hice, PhD**
> *Staff Fellow (Biologist)*
> *Division of Food Ingredients*
> **Center for Food Safety and Applied Nutrition**
> **Office of Food Additive Safety**
> **U.S. Food and Drug Administration**

stephanie.hice@fda.hhs.gov

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<2020-07-20 GRN 924 - Questions for Notifier.pdf>

Spherix Consulting Group

August 19, 2020

Stephanie Hice, PhD
Staff Fellow (Biologist)
Division of Food Ingredients
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 000924

Dear Dr. Hice:

In response to your email of July 20, 2020, below are our responses to your request for additional information regarding GRN 000924. FDA's questions are italicized text and our responses are in plain text. Additionally, we would like to alert you to a global error in the Notice regarding the production strain. The production strain *E. coli* BL21(DE3) #1242 is also known as JBT-2FL ΔlacZ, not JBT-2FL.

Regulatory:

1. On pg. 21, Jennewein states:

"The updated review now shows that the range of 2'-FL level in breast milk is greater than what was reported in GRN 000546. Thus, the proposed increase in use level in infant formula will accommodate the variations in breast milk 2'-FL levels resulting from ethnicity, secretor and Lewis-blood group status, lactation period, and term vs. preterm birth."

However, it is not clear from the notice on what basis Jennewein concluded that the new and higher use level of 3.64 g/L rather than 2.4 g/L is generally recognized as safe within the scientific community.

OFAS notes the following:

I. Permitted maximum use level in other regulatory authorities around the world ranges from 1.2 g/L (e.g. EU, FSANZ) to 2.0 g/L (e.g. Israel, Korea). Thus, 2.4 g/L is currently the highest maximum use level of 2'-FL in the world.

II. Notifiers for all previous GRNs of 2'-FL except Jennewein's GRN 000571 (e.g. GRNs 000546, 000650, 000735, 000749, 000852, 000897) have made the conclusion that the use level up to 2.4 g/L is GRAS; these conclusions were based on similar datasets portraying large variations in the levels of 2'-FL in breastmilk.

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http://spherixgroup.html

III. *It is widely recognized and accepted in the literature that the total level of HMOs as well as levels of many individual HMOs, including 2'-FL, decrease during period of lactation (Austin et al., 2019; Azad et al., 2018; Bode, 2012; Castanys-Munoz et al., 2013; Ray et al., 2019; Vandenplas et al., 2018). The highest levels of 2'-FL are typically found in colostrum, followed by transitional milk, and then mature milk (Erney et al., 2000; Kunz et al., 2017; Sprenger et al., 2017; Thurl et al., 2017). Given it is unlikely that infants expected to consume a non-exempt term infant formula would normally be consuming colostrum and/or transitional milk past 1st or 2nd week of feeding, the breastmilk reference for use level of 2'-FL in infant formula should reflect those of mature milk, not the levels found in colostrum or transitional milk.*

IV. *Variations in range, mean and median values of 2'-FL levels in reported breastmilk dataset can be impacted by the inclusion of samples from colostrum and transitional milk. Furthermore, data stratified according to secretor status (e.g. dataset from secretor only vs. pooled milk) also impacts mean and median values of 2'-FL. For example, as expected, analyses provided by the notifier of GRN 815 showed that dataset of mean values for 2'-FL levels were lower with "pooled" milk vs. "secretor" milk; these analyses also showed that compared to 2'-FL levels in "mature" milk (period of lactation >60 days), use level of 2.4 g/L is at or slightly above the mean values estimated from either pooled or secretor milk samples.*

V. *Based on information in the previous GRNs (000546, 000650, 000735, 000749, 000852, 000897) and our own searches, we are not aware of any relevant published literature indicating that the maximum use level of 2.4 g/L is inappropriate, nor literature providing compelling reasons or rationale as to why 3.64 g/L is more appropriate than the current use level.*

If Jennewein is aware of additional publicly available data and information, please provide a robust discussion as to how it supports that the proposed higher use level is generally recognized by the scientific community to be GRAS for use as an ingredient in cow's milk-based, non-exempt infant formula for term infants.

References

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

Azad, M.B., Vehling, L., Chan, D., Klopp, A., Nickel, N.C., McGavock, J.M., Becker, A.B., Mandhane, P.J., Turvey, S.E., Moraes, T.J., et al. (2018). Infant Feeding and Weight Gain: Separating Breast Milk From Breastfeeding and Formula From Food. Pediatrics 142.

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

Castanys-Munoz, E., Martin, M.J., and Prieto, P.A. (2013). 2'-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutr Rev 71, 773-789.

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

Kunz, C., Meyer, C., Collado, M.C., Geiger, L., Garcia-Mantrana, I., Bertua-Rios, B., Martinez-Costa, C., Borsch, C., and Rudloff, S. (2017). Influence of Gestational Age, Secretor, and Lewis Blood Group Status on the Oligosaccharide Content of Human Milk. J Pediatr Gastroenterol Nutr 64, 789-798.

Ray, C., Kerketta, J.A., Rao, S., Patel, S., Dutt, S., Arora, K., Pournami, F., and Bhushan, P. (2019). Human Milk Oligosaccharides: The Journey Ahead. Int J Pediatr 2019, 2390240.

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

Thurl, S., Munzert, M., Boehm, G., Matthews, C., and Stahl, B. (2017). Systematic review of the concentrations of oligosaccharides in human milk. Nutr Rev 75, 920-933.

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

Jennewein manufactures infant formula ingredients and conventional food ingredients, not infant formulas, and has determined the subject of GRN 000924 as Generally Recognized As Safe (GRAS) under the conditions of use based on generally available and accepted information. Importantly, the standard for determining the GRAS status of a substance is reasonable certainty of no harm. Additionally, per Section 412 of the Federal Food, Drug, and Cosmetic Act, all infant formula manufacturers must notify the U.S. FDA at least 90 days before marketing their infant formula, specify the quantitative formulation of each form of the infant formula, and provide assurance that the infant formula will not be marketed unless the formula meets the requirements of Section 412 of the Federal Food, Drug, and Cosmetic Act. Based on these regulatory tenets, it is therefore responsibility of the infant formula manufacturer to demonstrate that a new formula, which would include a new level of an oligosaccharide, will be appropriate and well tolerated. Thus, it is the responsibility of the infant formula manufacturer to demonstrate that a maximum use level of 2.4 g 2’-FL/L in an infant formula is

appropriate or inappropriate, and/or provide a rationale as to why 3.64 g/L is appropriate or inappropriate.

As stated in GRN 000924, the safety of the proposed use level of 2'-FL in infant formulas is based on publicly available and peer-reviewed studies that have quantitated the levels of 2'-FL in human milk and demonstrated that ingestion of 2'-FL at levels up to and including the use levels stated in GRNs 000546, 000650, 000735, 000749, 000852 and 000897 and the proposed use level stated in GRN 000924 are not associated with adverse outcomes on growth or health. Additionally, the information cited in GRN 000924 reiterates the general recognition that the ingestion of oligosaccharides, such as galactooligosaccharides (GOS), by infants from formula at levels up to 7.2 g/L is safe.

Importantly, the weight of evidence used to document general recognition of safety for oligosaccharides, including GOS, polydextrose, oligofructose, long chain inulin and fructooligosaccharides, which are surrogates for human milk oligosaccharides, is supported by a combination of data and studies. This evidence includes not only the levels of human milk oligosaccharides, which range from approximately 20 g/L in colostrum to 12 g/L in mature milk (Kunz et al., 1999; Kunz et al., 2000), but also toxicology studies in rats, tolerance studies in neonatal piglets, and clinical studies. The proposed use level of 2'-FL has been determined to be GRAS using the same evidence base and safety standard as what has been used to support GOS (and other oligosaccharides); GOS is GRAS for use in infant formulas at 7.2 g/L, exceeding the proposed use level for 2'-FL. Thus, based on the publicly available, peer-reviewed data and the precedent established for general recognition of safety for oligosaccharides in infant formula, which includes documentation of the range of levels of human milk oligosaccharides, rodent toxicology studies and neonatal piglet data, Jennewein concludes that the use of 2'-FL in infant formula at the proposed use level is GRAS.

References

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

Kunz, C., Rudloff, S., Baier, W., Klien, N., and Strobel, S. (2000). Oligosaccharides in Human Milk: Structural, Function, and Metabolic Aspects. In Annual Review of Nutrition, eds. (Indianapolis: Annual Reviews), pp. 699–722.

Chemistry:

2. *The notifier incorporates information and data from GRN 000571 and its supplement by reference, including the following: 1) specifications for 2'-FL, 2) respective analytical methods used to determine compliance with the specifications, and 3) results of batch analyses. For the administrative record, please provide a table of updated specifications along with the respective analytical methods and results from minimum three (preferably five) non-consecutive batch analyses to demonstrate that 2'-FL meets the established specifications. In addition, please confirm that all analytical methods used to test for each specification parameter are validated for that purpose.*

   To ensure a consistent food-grade product that is free of residues derived from the production strain, each batch of 2'-FL manufactured with the production strain JBT-2FLΔLacZ is evaluated against the same product specifications that were established in GRN 000571 (Table 1). These product specifications control the amount of 2'-FL, carbohydrate by-products, DNA and endotoxin residues derived from the production strain, heavy metals, and selected microbes. Each parameter is measured using the same, fit-for-purpose, compendial and/or internally validated methods that were used and determined to be GRAS in GRN 000571. Data from five batches of the finished ingredient show that the manufacturing process continues to reproducibly produce a product that meets the specifications that were established in GRN 000571.

Table 1. Product Specifications and Batch Data 2'-FL

Parameter	Analytical method	Specification	Batch number				
			16130039	16116049	16151039	26108010	26120020
Physical Parameters							
Appearance (Color)[4]	Visual	White to ivory-colored	Complies	Complies	Complies	Complies	Complies
Appearance (Form)[4]		Spray-dried powder	Complies	Complies	Complies	Complies	Complies
Chemical Parameters							
2'-Fucosyllactose	HPAEC-PAD	≥ 90 % (%DW)	92.2	98.4	95.5	97.8	94.9
Lactose		≤ 5 % (% Area)	1.1	< 0.5	2.5	< 0.5	0.5
3-Fucosyllactose		≤ 5 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Difucosyllactose		≤ 5 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Fucosylgalactose		≤ 3 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	0.5
Glucose		≤ 3 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Galactose		≤ 3 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Fucose		≤ 3 % (% Area)	0.7	< 0.5	1.8	< 0.5	0.7
Protein content[4]	Nanoquant (modified Bradford)	≤ 100 µg/g	< 10	< 10	< 10	< 10	< 10
Ash[1]	ASU L 06.00-4	≤ 0.5 %	< 0.01	0.03	0.08	< 0.01	0.08
Moisture[4]	KF titration	≤ 9.0 %	5.8	5.8	6.3	6.6	5.2
Endotoxins[3]	Ph. Eur. 2.6.14	≤ 300 EU/g	14	< 5	< 5	< 5	< 5
Aflatoxin M1[1]	DIN EN ISO 14501	≤ 0.025 µg/kg	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
GMO residues[2]	PCR	Negative	Negative	Negative	Negative	Negative	Negative
Heavy Metals							
Arsenic[1]	ASU L 00.00-135 – ICP-MS	≤ 0.2 mg/kg	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Cadmium[1]		≤ 0.1 mg/kg	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010
Lead[1]		≤ 0.02 mg/kg	< 0.010	< 0.010	0.020	< 0.010	< 0.010
Mercury[1]		≤ 0.5 mg/kg	< 0.005	0.007	< 0.005	< 0.005	< 0.005
Microbiology							
Standard Plate Count[1]	ISO 4833-2	≤ 10000 cfu/g	< 10	< 10	30	20	< 10
Yeast and Mold[1]	ISO 21527-2	≤ 100 cfu/g	< 20	< 20	< 20	< 20	< 20
Coliform	ISO 4832	Absent/11 g	Absent	Absent	Absent	Absent	Absent
Enterobacteriaceae[1]	ISO 21528-1	Absent/11 g	Absent	Absent	Absent	Absent	Absent

Table 1. Product Specifications and Batch Data 2'-FL

Parameter	Analytical method	Specification	Batch number				
			16130039	16116049	16151039	26108010	26120020
Salmonella [1]	ISO 6579	Absent/100 g	Absent	Absent	Absent	Absent	Absent
Cronobacter sakazakii[1]	ISO/TS 22964	Absent/100 g	absent	absent	absent	absent	absent

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

[1]Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory; ash limit of quantitation (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 = 5 EU/g.

[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.

3. *The notifier states that 2'-FL is manufactured using the same process as described in GRN 000571 and its supplement. In GRN 000571 (Appendix J), the notifier lists cobalt (II) chloride hexahydrate as a component of the fermentation medium used in the manufacture of 2'-FL. Per 21 CFR 189.120, food containing added cobaltous salts, including cobalt (II) chloride, is deemed adulterated. Please discuss the potential presence of cobalt in the final product and provide analytical data from three (preferably five) non-consecutive batches to demonstrate that cobalt is not present in the final product.*

   During manufacturing, the culture medium, which contains cobalt and variety of other elements required for the growth of the production strain JBT-2FL ΔlacZ, is subjected to ion exchange chromatography and electrodialysis to remove the elements from the finished product. To confirm that these processes remove cobalt, Jennewein quantitated the levels of cobalt in five batches of the finished ingredient (Table 2). Cobalt was below the limit of quantitation in all batches, indicating that the manufacturing process removes this key medium element from the finished ingredient.

Table 2. Cobalt Analysis of 2'-Fucosyllactose

Element[1]	Method	LOQ	Batch Number				
			16130039	16116049	16151039	26108010	26120020
Cobalt (mg/kg)	PV-347 (ICP-MS)	0.04 mg/kg	< 0.04	< 0.04	< 0.04	< 0.04	< 0.04

Abbreviations: ICP-MS, inductively coupled plasma mass spectrometry; LOQ, limit of quantitation
[1]Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory.

4. *The notifier states that the 2'-FL that is the subject of GRN 924 is the same as the subject of GRN 000571. GRN 000571 describes two formulations of 2'-FL, a powder and liquid concentrate. Please confirm that the 2'-FL in GRN 000924 also refers to the same two formulations and that the respective use levels of the formulations would be adjusted accordingly to achieve 3.64 g/L 2'-FL in the infant formula as consumed.*

   The 2'-FL that is the subject of GRN 000924 will be used in the same two formulations described in GRN 000571. To achieve 3.64 g/L 2'-FL in the infant formulas as consumed, the respective use levels of the formulations will be adjusted accordingly.

5. *On page 16 of the notice (Table 4), the notifier provides estimates of dietary exposure to 2'-FL expressed in g/day and in g/kg bw/day. According to the footnote to Table 4, the estimates in g/day were calculated by multiplying the estimates provided in GRN 000571 (Table 1) by the 1.82-fold increase in use level for 2'-FL. However, it is not clear how the notifier calculated the estimates in g/kg bw/day. We note that GRN 000571 does not provide estimates in g/kg bw/day. Please explain how the notifier calculated the estimates in g/kg bw/day provided in Table 4 of GRN 000924.*

   The estimates in g/kg bw/day were calculated by dividing the mean and 90th percentile EDIs in g/day by the mean body weights of participants who provided a body weight in the NHANES 2009-2010 survey and were in the mean and 90th percentile EDI groups.

For the infants 0 – 5 months old, the body weights ranged from 2.8 to 10 kg and the mean weights were 6.4 and 8.2 kg at the mean and 90th percentile EDIs, respectively. For the infants 6 – 11 months old, the body weights ranged from 6.1 to 12.5 kg and the mean weights were 9.1 and 11.1 kg at the mean and 90th percentile EDIs, respectively. A new Table 4, which now includes a new column that highlights the number of participants with a body weight consuming infant formula during the study period, is provided below.

Table 4. Estimated Daily Intake of Jennewein 2'-FL from Infant Formula

Population[b]	n[c]	n[d]	Percent users[e]	Mean		90th Percentile	
				g/day[a]	g/kg bw/day	g/d[a]	g/kg bw/day
Infants, 0-5 mo	141	141	100	3.3	0.52	4.6	0.56
Infants, 6-11 mo	142	140	86.3	2.9	0.32	4.0	0.36

Abbreviations: 2'-FL = 2'-fucosyllactose; d =day; g = gram(s); bw = body weight; mo = month(s).
Source: NHANES 2009-2010 Data; Jennewein Biotechnologie, GmbH.
[a]Values are 1.82-fold those noted in Table 1 of GRN 000571.
[b]Breastfeeding infants and children were excluded from the sample population.
[c]Number of participants consuming infant formula during the study period.
[d]Number of participants consuming infant formula during the study period with a body weight available.
[e]Weighted percent.
Note: Because this is a daily average, some participants who had day 1 but not day 2 data are included using a single day of consumption.

Microbiology:

6. *Please state whether Escherichia coli BL21(DE3) strain "JBT-2FL" (#1242) 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 DMSZ - German Collection of Microorganisms and Cell Cultures GmbH with the deposition number DSM 33609. The host strain from which JBT-2FL ΔlacZ 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 2'-FL.

7. *Please state whether E. coli BL21(DE3) strain "JBT-2FL" (#1242) is non-toxigenic.*

JBT-2FL ΔlacZ is non-toxigenic.

8. *The notifier provides specifications for Salmonella serovars and Cronobacter sakazakii listed as negative by test in 100 grams. In GRNs 000921-000923 and in GRN 000925, the notifier provides specifications for Salmonella serovars and C. sakazakii listed as negative by test in 25 grams and 10 grams, respectively. For the administrative record, please clarify this discrepancy.*

The specifications for *Salmonella* serovars and *Cronobacter* sakazakii for the 2'-FL that is the subject GRN 000924 were established and determined GRAS in 2015 in GRN 000571. Although these specifications have remained in place since then, Jennewein has learned that specifications for *Salmonella* serovars and *Cronobacter sakazakii* of absent in 25 g product and absent in 10 g of product, respectively, are sufficient to produce a safe infant formula ingredient. Based on these results, the specifications for *Salmonella* serovars and *Cronobacter sakazakii* of absent in 25 g product and absent in 10 g of product were established for the subjects of GRNs 000921-000923 and GRN 000925. However, because the *Salmonella* serovars and *Cronobacter sakazakii* specifications for the 2'-FL that is the subject of GRN 000924 are based on GRN 000571, Jennewein decided to continue with the specifications that were established in GRN 000571, despite the stricter acceptance criteria.

9. *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-2FL" (#1242) has the same virulence profile.*

   Because Jennewein engineered JBT-2FL ΔlacZ with genes with known functions and that do not confer virulence using site-specific homologous recombination or transposition, JBT-2FL ΔlacZ has the same virulence profile as *E. coli* BL21(DE3).

10. *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-2FL" (#1242) is expected to result in any safety concerns.*

    Because Jennewein used E. *coli* BL21(DE3), a strain widely used to manufacture food and pharmaceutical ingredients, as the host strain for engineering JBT-2FL ΔlacZ, and genetic elements that encode proteins with known functions and do not encode invasion factors, adhesion molecules, and enterotoxins associated with virulence via site-specific homologous recombination or transposition, JBT-2FL ΔlacZ is not expected to result in any safety concerns.

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

Dietrich B. Conze, Ph.D.
Managing Partner

From:	Dietrich Conze
To:	Hice, Stephanie
Cc:	Claire Kruger; Kathy Brailer
Subject:	Re: GRN 000924 - Request for Teleconference
Date:	Monday, October 5, 2020 6:04:54 PM

Hi Stephanie,
I just wanted to follow-up to my previous email and alert you to an error in the letter from Dr. Jennewein. The references to the mean and median in the figure stated in the figure legend are wrong. The correct legend should read the following, which agrees with the values stated in the text of the letter:

Figure legend: The distribution, mean, median, standard deviation, and 90% confidence internal of the mean levels of 2'-FL in human milk obtained from secretor mothers reported in published literature. One hundred and eleven means were abstracted from the studies reviewed by Thurl et al. (2017) as well as other studies published since Thurl et al. (2017). All of the new studies met the inclusion criteria used by Thurl et al, except that when only medians were reported, the medians were used in the calculations. The histogram depicts the distribution of the 2'-FL means and the corresponding location of the mean (red line), median (green line), the lower 68% confidence interval and the upper 69% confidence interval (box with dotted line), and the 90% confidence internal (blue dashed line).

I apologize for the confusion.
Regards.
Dietz

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Biotechnologie GmbH

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College Park, MD 20740

RE: **Questions Regarding GRN 000924**

October 5th, 2020

Dear Dr. Hice,

We appreciate the opportunity to meet with the agency on October 9 to discuss GRN 000924. In advance of that meeting and for the purposes of facilitating discussion, we wanted to respond to certain items raised in your email of September 14, 2020. As described below, we continue to believe that the submission meets the GRAS standard set forth in 21 U.S.C. § 321(s) and 21 C.F.R. § 170.30. More importantly, it is unclear why the average level of 2'-FL in human milk is relevant in determining whether the level proposed in the notice, 3.64 g/L, is generally recognized as safe for its intended use. So long as the level proposed has been demonstrated by scientific evidence to be safe for its intended use (*i.e.*, to not be harmful under the conditions of its intended use) and is generally recognized by qualified experts in the field to be safe, the GRAS standard is met. That is true regardless of the average level of 2'-FL in human milk.

Under 21 C.F.R. § 106.96, infant formula manufacturers have a separate responsibility to demonstrate the suitability or appropriateness of infant formula products, which includes careful consideration of the totality of the ingredients[1]. However, suitability is and should be evaluated as part of the Infant Formula Notification process, not as part of the GRAS review process.

We continue to believe that GRN 000924 meets the GRAS standard and that we have demonstrated, with reasonable certainty, that the substance is not harmful under the conditions of its intended use. As described in more detail below, with previous GRAS notices for oligosaccharides used in infant formula, FDA did not have questions about the GRAS status of the ingredients when appropriate manufacturing controls, food grade specifications, well-conducted rodent studies confirming lack of toxicity at relevant levels of exposure, and neonatal piglet studies that are surrogates for tolerance and safety in infants were used. In the case of 2'-FL at 3.64 g/L, these requirements were satisfied in GRN 000924. In addition, corroborative information regarding the range of naturally occurring levels of oligosaccharides that are present in human milk demonstrates that the proposed level falls within the established range of human milk. For reference, the mean, median, the lower 68% confidence interval, upper 69% confidence interval, and 90% confidence internal level of the means for 2'-FL levels

[1] Infant formula manufacturers are required under 21 C.F.R. § 106.96 to meet the quality factors of (1) normal physical growth with a growth monitoring study of infants and (2) sufficient biological quality of protein with a protein efficiency ratio (PER) rat bioassay.

quantified in human milk are 2.89, 2.66, 1.83, 3.92, and 4.41 g/L based on our updated analysis of the published literature (see Figure).

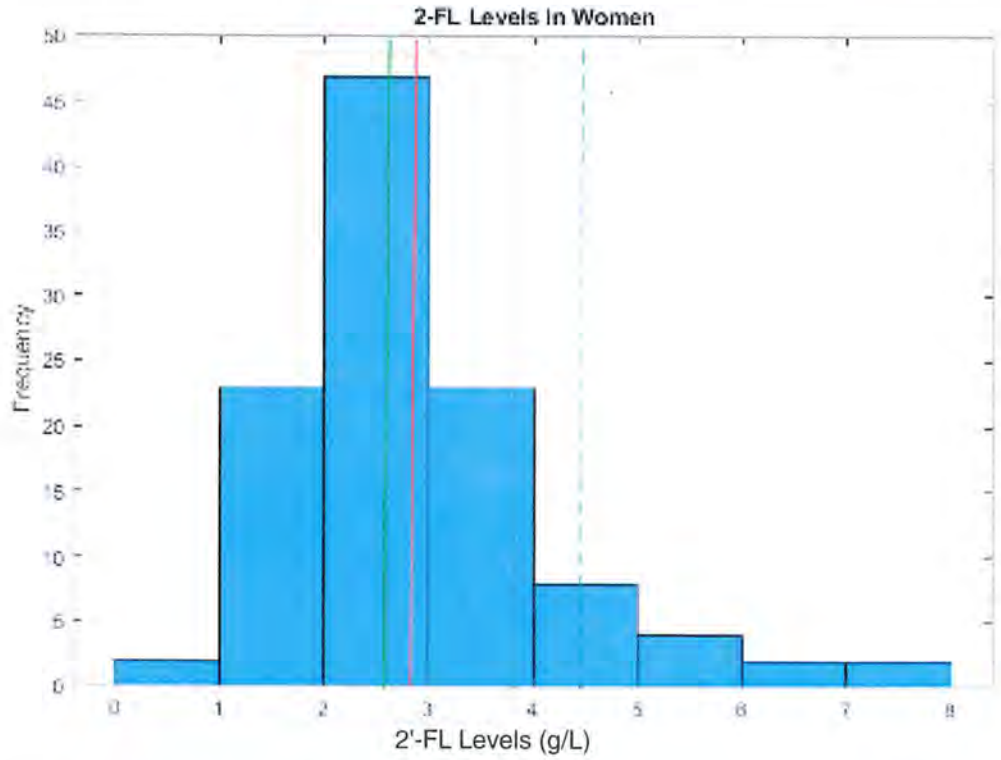

Figure legend: The distribution, mean, median, standard deviation, and 90% confidence internal of the mean levels of 2'-FL in human milk obtained from secretor mothers reported in published literature. One hundred and eleven means were abstracted from the studies reviewed by Thurl et al. (2017) as well as other studies published since Thurl et al. (2017). All of the new studies met the inclusion criteria used by Thurl et al, except that when only medians were reported, the medians were used in the calculations. The histogram depicts the distribution of the 2'-FL means and the corresponding location of the mean (green line), median (red line), the lower 68% confidence interval and the upper 69% confidence interval (box with dotted line), and the 90% confidence internal (blue dashed line).

FDA questions from July 20, 2020 reference that there is no literature supporting why 3.64 g/L is a more appropriate level than 2.4 g/L. However, that is not the standard. Indeed, if FDA were to take the position that 2'-FL is only GRAS at the average level in human milk (2.4 g/L), that would necessarily mean that human milk with levels above the average is unsafe for infants. The review of all publicly-available data, described above, did not identify any information that suggested 2'-FL would be harmful when used in infant formula at levels up to 3.64 g/L. The *suitability* of an infant formula containing 2'-FL up to 3.64 g/L (and other ingredients, including other oligosaccharides) will be demonstrated through the quality factors of normal physical growth and sufficient biological quality of protein during the Infant Formula Notification process.

For ease of reference, we provide in this letter a summary of the evidence presented in GRN 000924 supporting reasonable certainty that 2'-FL is not harmful under the proposed use at 3.64 g/L in infant formula. The safety studies provide the certainty that 2'-FL is not harmful under the proposed conditions of use, although information about the range of 2'-FL concentrations in human milk, and thus demonstration of exposure to these levels, corroborates this conclusion.

Jennewein
Biotechnologie GmbH

In 2015, Jennewein Biotechnologie received an FDA no questions letter for the use of its 2'-FL product in infant formula at a level of 2.0 g/L (GRN 000571). At the same time, Glycom also received a no questions letter for the use of their 2'-FL product in infant formula at a similar level of 2.4 g/L (GRN 000546). In both GRAS Notices, the intended use levels were determined by 90-day rat toxicology studies, and surveying the published literature to establish the range of 2'-FL that naturally occurred in breast milk to corroborate the findings of the toxicology studies. For the level of 2'-FL in breast milk, Jennewein Biotechnologie found that the levels of 2'-FL ranged from 2.4 to 4.1 g/L from a variety studies published up to 2010. Glycom found, from a variety of studies published from 1997 to 2014, that although it may be present at levels up to 7.0 g/L, 2'-FL is generally present in breast milk from 1.1 to 4.2 g/L. Since then, numerous 2'-FL products produced by other manufacturers have also been determined GRAS and they all have referenced the range of 2'-FL in human milk as corroborative evidence to support the GRAS determinations.

The 2'-FL product that is the subject of GRN 000924 is also the subject of GRN 571. The safety of 2'-FL in GRN 571 was demonstrated through a neonatal piglet study on 2'-FL alone (NOAEL of 0.29 g/kg/day), a 90-day oral toxicology study on 2'-FL alone (NOAEL of 7.6 g/kg/day (males) and 8.72 g/kg/day (females)), and genotoxicity studies on 2'-FL alone. In GRN 924, additional data was provided to demonstrate the safety of the proposed use of 2'-FL at 3.64 g/L, including a 90-day toxicology study of an HMO Mix comprising 52% 2'-Fucosyllactose, 13% 3-Fucosyllactose, 26% LNT, 5% 6'-SL and 4% 3'-SL (NOAEL of 5.67 g/kg/day (males) and 6.97 g/kg/day (females)), a neonatal piglet study with the same HMO mixture (NOAEL of 3.58 g/kg (males) and 3.66 g/kg (females)), as well as genotoxicology studies for the HMO mixture. All of these studies are either summarized or incorporated by reference in the GRAS notice. For GRN 000924, we reviewed 21 studies that have been published since 2014 to determine that the intended use level of 3.64 g/L is within the range of 2-FL in human milk, corroborating the safety demonstrated in these studies. The results from the literature review revealed that the levels of 2'-FL in breast milk range from 0 to 9.5 g/L with means and medians ranging from 0.01 to 4.6 and 0.01 to 5.2 g/L, respectively.

It is important to note that galactooligosaccharides (GOS) and fructooligosaccharides (FOS), both synthetic surrogates for human milk oligosaccharides, have been widely used in infant formulas at concentration of up to 8.0 g/L and the safe use in infant formulas is supported by GRAS Notices 000233, 000236, 000285, 000334, 000484, 000489, 000495, 000518, 000569, 000620, 000671, 000721, and 000729 (GOS), as well as 000044, 000537, 000623, 000717, and 000797 (FOS). Similarly, there are other ingredients that have been the subject of GRAS notifications (to which FDA "had no questions") when added at concentrations that may have exceeded the mean concentration in human milk but matched the performance of breastfed infants. For example, crystalline / suspended lutein was clinically demonstrated to have a reduced bioavailability from milk-based infant formulas compared to human milk (e.g., GRN 000221, 000390) (Bettler et al. 2010 and Mackey et al. 2012).

The Panel of Experts that reviewed GRN 000924 concur that addition of up to 3.64 g/L 2'-FL in infant formula is GRAS, meeting the criteria of reasonable certainty of no harm under this proposed use. The suitability of infant formula products containing 2'-FL up to 3.64 g/L will be demonstrated by infant formula manufacturers intending to market infant formula products using this oligosaccharide.

We are willing to discuss open questions during our conference call on October 9th.

Sincerely,

[redacted]

Dr. Stefan Jennewein (CEO, Jennewein Biotechnologie GmbH)