Spherix Consulting Group

March 19, 2020

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

Dear Dr. Morissette:

It is our opinion that the enclosed GRAS Determination for the Use of 3-Fucosyllactose (3-FL) in Non-Exempt Term Infant Formula constitutes a new notification because 3-FL is novel food ingredient.

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 Use of 3-Fucosyllactose (3-FL) in Non-Exempt Term Infant Formula, and all references

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GRAS Determination for the Use of 3-Fucosyllactose in Non-Exempt Term Infant Formula

Prepared for:

Jennewein Biotechnologie GmbH
Maarweg 32
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Germany

Prepared by:

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March 19, 2020

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LIST OF ABBREVIATIONS

2'-FL: 2'-Fucosyllactose

3-FL: 3-Fucosyllactose

3'-SL: 3'-Sialyllactose

6'-SL: 6'-Sialyllactose

Alb: Albumin

ALT: Alanine aminotransferase

araA: Arabinose isomerase

BMI: Body mass index

BW: Body weight

CBPI: Cytokinesis-block proliferation index

CFR: United States Code of Federal Regulations

CFU: Colony forming units

CHO: Chinese hamster ovary cells

CI: Confidence interval

COSY: Correlation spectroscopy

DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen

DW: Dry weight

EDI: Estimated Daily Intake

EFSA: European Food Safety Authority

EU: Endotoxin unit

F6PPK: Fructose-6-phosphate phosphoketolase

FCC: Food Chemicals Codex

FDA: United States Food and Drug Administration

FFDCA: Federal Food, Drug, and Cosmetic Act

FOIA: Freedom of information Act

FOS: Fructooligosaccharides

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

Fru-6-P: Fructose-6-phosphate

FSSC: Food Safety System Certification

FUT: Fucosyltransferase

GI: Gastrointestinal

Glc-1-P: Glucose-1-phosphate

Glc-6-P: Glucose-6-phosphate

Gln-1-P: Glucosamine-1-phosphate

Gln-6-P: Glucosamine-6-phosphate

Glob: Gobulin

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

GMO: Genetically modified organism

GMP: Good manufacturing practices

GOS: Galactooligosaccharides

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

HCD: Historical control data

HDL-C: high-density lipoprotein cholesterol

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

HMO: Human milk oligosaccharides

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

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

ICP-MS: Inductively coupled plasma mass spectrometry

IFNγ: Interferon gamma

LC-MS: Liquid chromatography coupled with mass spectrometry

LDL-C: Low-density lipoprotein cholesterol

LDPE: Low-density polyethylene

LNDFHI: lacto-N-difucohexaose I

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

LNT: Lacto-N-tetraose

LOD: Limit of detection

LOQ: Limit of quantitation

MCH: Mean corpuscular hemoglobin

MCV: Mean corpuscular volume

ND: Not detected

NHANES: National Health and Nutrition Examination Surveys

NIH: National Institutes of Health

NMR: Nuclear Magnetic Resonance

NOAEL: No Observed Adverse Effect Level

OECD: Organization for Economic Cooperation and Development

PCR: Polymerase chain reaction

Ph Eur: European Pharmacopoeia

pLNnH: Para-lacto-N-neohexaose

qPCR: Quantitative polymerase chain reaction

RI: Replicative index

TP: Total protein

UDP-Gal: UDP-galactose

UDP-Glc: UDP-glucose

UDP-GlcNAc: UDP-N-acetylglucosamine

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

A. SUBMISSION OF GRAS NOTICE

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

B. NAME AND ADDRESS OF THE SPONSOR

Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

C. COMMON OR USUAL NAME

3-Fucosyllactose (3-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 3-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 3-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 3-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 3-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 % 3-FL dry weight.

    a. 3-Fucosyllactose is a neutral, fucosylated oligosaccharide in human milk.

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

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

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

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

    f. Fermentation by-products include fucose and lactose, which are known components of human milk; their presence in the finished ingredient is not of toxicological concern.

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

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

2. Human milk oligosaccharides, including 3-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 3-FL in breast milk ranges from 0 to 5.9 g/L, with means and medians ranging from 0 to 2.4 and 0 to 1.1 g/L, respectively.

4. Genotoxicology and subchronic toxicology studies published by Pitt et al. (2019) show that 3-FL is not genotoxic and has a NOAEL (no observed adverse effect level) of 10% of the diet in rats, which was the highest level addition level tested and equivalent to 5.98 and 7.27 g/kg bw/day for males and females.

5. The addition of 0.91 g/L 3-FL in infant formula will result in an intake of approximately 0.64 g/day (146 mg/kg/day) for a 1-month-old infant and 0.88 g/day (115 mg/kg/day) for a 6 month-old infant.

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

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

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

    c. The qualitative comparability and quantitative similarity of the Jennewein 3-FL to the 3-FL ingredient tested by Pitt et al. (2019).

    d. Corroborative genotoxicology and 90-day subchronic dietary toxicology studies conducted with a mixture of human milk oligosaccharides published by Parschat et al. (2020), which contained 16% (dry weight) of Jennewein's 3-FL);

    e. A corroborative unpublished tolerance study in neonatal piglets conducted with a mixture of HMOs containing up to 0.8 g/L of Jennewein-manufactured 3-FL study that showed an HMO mixture containing 3-FL was well-tolerated and supported normal growth in neonatal piglets.

Therefore, 3-FL is safe and GRAS at the proposed level of addition to the intended infant formula. 3-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] 19/3/20

Signature of Authorized Representative of Jennewein Biotechnologie GmbH

Date

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

A. COMMON OR USUAL NAME

3-Fucosyllactose (3-FL; CAS No. 41312-47-4)

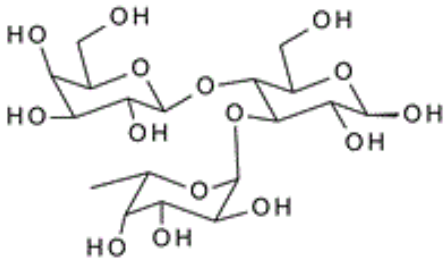
B. CHEMICAL NAME

6-deoxy-α-L-galacto-hexopyranosyl-(1→3)-β-D-galacto-hexopyranosyl-(1→4)-β-D-gluco-hexopyranose

C. MOLECULAR FORMULA AND MASS

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

D. STRUCTURAL FORMULA



E. DESCRIPTION OF 3-FUCOSYLLACTOSE

3-Fucosyllactose (3-FL) is a fucosylated, neutral trisaccharide composed of L-fucose, D-galactose, and D-glucose units. It is a naturally occurring oligosaccharide found in human milk. 3-FL is produced by fermentation using a genetically engineered strain of *Escherichia coli* BL21(DE3). It is then purified from the culture medium and spray-dried into a powder with a purity of ≥ 90%. Residual impurities include lactose and other carbohydrate by-products. Importantly, the structure of 3-FL produced by fermentation has been shown to be consistent with the structure of 3-FL as confirmed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), ^{1}H nuclear magnetic resonance spectroscopy (NMR), ^{13}C NMR, double-quantum filtered $^{1}H^{1}H$ correlation spectroscopy (COSY), phase-sensitive $^{1}H^{13}C$-heteronuclear single quantum correlation spectroscopy (HSQC), and phase-sensitive $^{1}H^{13}C$-heteronuclear multiple bond correlation spectroscopy (HMBC).

F. PRODUCTION PROCESS

3-FL is produced by fermentation using *JBT-3FL*, a genetically engineered strain of *E. coli* BL21(DE3). Following synthesis, 3-FL is purified from the fermentation medium. The resulting 3-FL concentrate is dried into a powder.

1. Description of the Production Strain

JBT-3FL is a genetically engineered strain of *E. coli* BL21(DE3). *JBT-3FL* was generated in a manner similar to *JBT-2FL*, which is used to produce the 2'-fucosyllactose (2'-FL) that is the subject of GRN 571. Therefore, the details of the engineering summarized in GRN 571 are incorporated by reference (pages 6 and 7; Appendix K). Briefly, a strain known as the Basic strain was engineered first, starting with the parental strain *E. coli* BL21(DE3). All genes integrated into the Basic strain are well characterized, and the strain does not carry plasmids or episomal vectors. The Basic strain was further engineered to generate the production strain, *JBT-2FL*, which also expresses an α-1,2-fucosyltransferase and a heterologous exporter to facilitated 2'-FL export out of the cells. In the case of *JBT-3FL*, the same Basic strain used in the design of *JBT-2FL* was further engineered to express genes specific to the production of 3-FL.

Both the Basic Strain and *JBT-3FL* production strain are stored at the production site as glycerol stocks in a master cell bank at -80°C. Both strains will be deposited at the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen)-German Collection of Microorganisms and Cell Cultures. The glycerol stocks are used to produce working cell banks, which are then used to the production of the finished ingredient.

a. The Basic Strain

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

homologous recombination or transposition and used for selection of the recombinants were then removed from the genome by plasmid and Cre-mediated recombination (Lambert et al., 2007; Hoess and Abremski, 1990). All gene deletions and insertions were verified by PCR using oligonucleotides specific to the coding sequence and basic strain genomic DNA. Loss of the plasmids used to express λ red recombinase, transposase and Cre recombinase, all of which contained ampicillin resistance genes and temperature-sensitive origins of replication, was confirmed by ampicillin sensitivity after incubation at 42°C, and failure to amplify plasmid specific DNA.

Table 1. Genetic Manipulations in the Basic Strain

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

b. The Production Strain, JBT-3FL

In the case of *JBT-3FL*, the Basic strain was further engineered to express a 1,3-fucosyltransferase to allow for the production and secretion of 1,3-fucosyllactose from lactose. Specifically, an endogenous partial β-galactosidase gene was deleted by homologous recombination and a phosphomannomutase, mannose-1-phosphate guanosyltransferase, GDP-fucose-4,6-dehydrase, and GDP-fucose synthase from *E. coli* K12, and an α-1,3-fucosyltransferase from *Bacteriodes fragilis* NTCT9343 were integrated into the Basic strain by transposition (Table 2). The DNAs encoding the phosphomannomutase, mannose-1-phosphate guanosyltransferase, GDP-fucose-4,6-dehydrase, and GDP-fucose synthase were amplified from *E. coli* K12 genomic DNA. The DNA encoding the 1,3-fucosyltransferase was synthesized de

novo. Additionally, two antibiotic resistance genes, dihydrofolate reductase and bleomycin resistance protein, were synthesized de novo and integrated along with the *E. coli* K12 and *B. fragilis* NTCT9343 genes to facilitate selection of the recombinants. The integrants were then subjected to nitrosoguanidine (NTG) mutagenesis and screened for their ability to produce high levels of 3-FL, resulting in *JBT-3FL*. All gene insertions were then verified by PCR using oligonucleotides specific to the coding sequence. Loss of the plasmids used to express the transposase, which contained an ampicillin resistance gene and temperature-sensitive origin of replication, was confirmed by growth at 42°C, ampicillin sensitivity, and failure to amplify plasmid specific DNA. All integrated genes remain in the genome and, although *JBT-3FL* possesses the dihydrofolate reductase and bleomycin resistance genes used for integrant selection, no plasmids or other episomal vectors remain in the genome.

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

Gene Product Name	Origin of the Gene	Manipulation	Effect
5'- β-galactosidase	*E. coli* BL21(DE3)	Deletion	No effect (removal of a gene that is non-functional due to a truncation)
Phosphomannomutase	*E. coli K12*	Ectopic expression	To allow for sufficient GDP-fucose production
Mannose-1-phosphate guanosyltransferase	*E. coli K12*	Ectopic expression	
GDP-fucose-4,6-dehydratase	*E. coli K12*	Ectopic expression	
GDP-fucose synthase	*E. coli K12*	Ectopic expression	
α1,3-fucosyltransferase	*Bacteroides fragilis NTCT9343*	Ectopic expression	To enable for fucosylation of lactose
Antibiotic resistance genes			
Dihydrofolate reductase conferring resistance to trimethoprim	*Citrobacter freundii*	Ectopic expression	To allow for the selection of recombinants during genetic engineering
Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	Ectopic expression	

2. Manufacturing

a. Quality

Production of 3-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 a FDA-registered Food Facility (Registration # 1303109037512). Production also occurs at other Jennewein-qualified manufacturers that are GMP-, ISO-, and International Featured Standards Food 6.1-compliant via third party audits.

b. Processing Aids and Food Contact Substances

All raw materials, processing aids, and food contact substances used to produce the 3-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 (pg. 17; Appendix E, pg. 99-144; Appendix J, pg. 280-281) are incorporated by reference. Additional processing aids comply with European Pharmacopoeia, United States Pharmacopeia-National Formulary (USP-NF), or Japanese Pharmacopoeia specifications or appropriate product monographs. The water used throughout the manufacturing process complies with the TrinkwV, 2001 in Germany and the Council Directive 98/83/EC in the European Union and is non-fluoridated drinking water. All food contact surfaces (fermentation vessels and packaging materials) are either stainless steel or comply with the conditions of use that are specified in the US Code of Federal Regulations. The final product is packaged in food grade paper/low-density polyethylene (LDPE) bags in compliance with 21 CFR §177.1520. None of the processing aids are recycled or reused.

c. Production

Except for certain process parameters, 3-FL is manufactured using the same process as the 2'-FL that is the subject of GRN 571, which received a "no questions" letter from FDA. The detailed summary of the production process provided in GRN 571 (pg. 6-9) is therefore incorporated by reference. Briefly, the production of 3-FL involves three steps (Figure 1). During Step 1, the engineered strain of *E. coli* BL21(DE3), *JBT-3FL*, is expanded in minimal media containing a carbon source (glucose, sucrose, 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 3-FL into the culture medium. Step 2 involves purification of the oligosaccharide from the culture medium. Lactase may be added at the end of the process to degrade excess lactose. Step 3 involves spray-drying of the 3-FL concentrate product, producing a 3-FL powder.

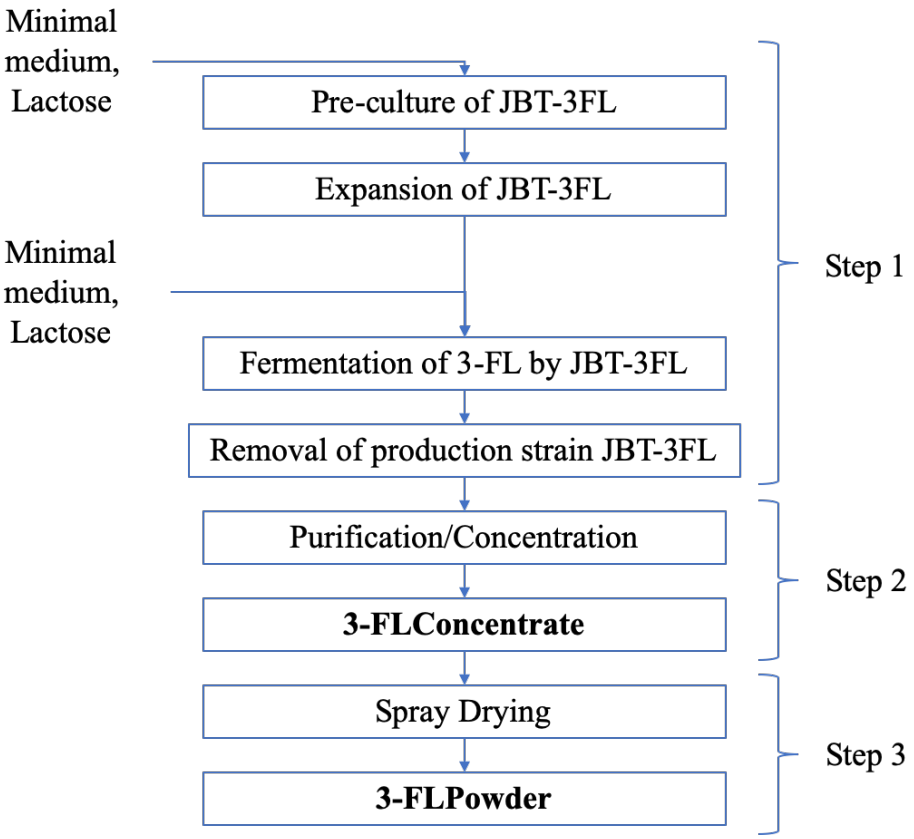


Figure 1. Production Process for 3-Fucosyllactose

A genetically modified strain of *E. coli* BL21(DE3) *JBT-3FL* is expanded in minimal medium to generate 3-fucosyllactose (3-FL) using lactose as substrate. 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 3-FL concentrate. Finally, the concentrate is spray dried to generate the 3-FL powder.

G. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

1. 3-FL Product Specifications and Batch Data

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

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

Table 3. Final Product Specifications and Batch Data for 3-Fucosyllactose

Parameter	Analytical method	Specification	Batch number		
			16121019	16121029	16121039
Physical Parameters					
Appearance (Color)[4]	visual	white to ivory-colored	Complies	Complies	Complies
Appearance (Form)[4]		spray-dried powder	Complies	Complies	Complies
Chemical Parameters					
3-Fucosyllactose[4]	HPAEC-PAD	≥ 90%	96.3	96.9	96.5
Lactose[4]		≤ 5%	0.7	0.5	0.7
Glucose[4]		≤ 3%	< LOQ	< LOQ	< LOQ
Galactose[4]		≤ 3%	< LOQ	< LOQ	< LOQ
Fucose[4]		≤ 3%	1.1	0.6	< LOQ
Protein[4]	Nanoquant (modified Bradford)	≤ 100 µg/g	< LOQ	< LOQ	< LOQ
Ash[1]	ASU L 06.00-4	≤ 1.0 %	0.36	0.33	0.25
Moisture[4]	KF titration	≤ 9.0 %	6.7	7.4	7.7
Endotoxins[3]	Ph. Eur. 2.6 14, method C	≤ 10 EU/mg	0.007	0.009	0.023
Aflatoxin M1[1]	DIN EN ISO 14501	≤ 0.25 µg/kg	< LOQ	< LOQ	< LOQ
GMO residues[2]	qPCR	Negative	Negative	Negative	Negative
Heavy Metals					
Arsenic[1]	ASU L 00.00-135 – ICP-MS	≤ 0.2 mg/kg	ND	ND	ND
Cadmium[1]		≤ 0.1 mg/kg	ND	ND	ND
Lead[1]		≤ 0.02 mg/kg	ND	ND	ND
Mercury[1]		≤ 0.5 mg/kg	ND	ND	ND
Microbes					
Standard Plate Count[1]	ISO 4833-2	≤ 10000 cfu/g	< 10	< 10	< 10
Yeast and Mold[1]	ISO 21527-2	≤ 100 cfu/g	< 20	< 20	< 20
Enterobacteriaceae[1]	ISO 21528-1	≤ 10 cfu/g	< 10	< 10	< 10
Salmonella[1]	ISO 6579	Absent/25 g	Absent	Absent	Absent
Cronobacter sakazakii[1]	ISO/TS 22964	Absent/10g	Absent	Absent	Absent

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

2. Other Quality Attributes

a. Elemental analysis

Although the oligosaccharide-containing media is subjected to ion exchange chromatography and electrodialysis to minimize the elements in the finished product, Jennewein analyzed three batches of the finished 3-FL product for the levels of manganese, selenium, iron, copper, molybdenum, nickel, zinc, and cobalt (Table 4). Importantly, manganese and molybdenum, which are all media components, were all below the limit of detection. Although cobalt was detected in two of the batches, the levels were at or close to the level of quantitation. The amount of cobalt consumed by a one- and six-month-old infant from the proposed use level in infant formula is 0.009 and 0.006 µg/kg/day (assuming that the average body weight of a one- and six-month old infant is 3.3 and 7.61 kg, respectively), which is below the provisional reference dose of 0.3µg/kg/day established by the Environmental Protection Agency (EPA) (US EPA, 2008). To ensure that the manufacturing process continues to produce a high-quality finish ingredient, these analyses will be conducted on an annual basis.

Table 4. Elemental Analysis of 3-Fucosyllactose

		16121019	**16121029**	**16121039**
Manganese (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 1.7	< 1.7	< 1.7
Selenium (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.094	0.069	0.025
Iron (mg/kg)	ASU L 00.00-135 (ICP-MS)	1.2	1.1	0.75
Copper (mg/kg)	ASU L 00.00-135 (ICP-MS)	1.8	1.2	2.3
Molybdenum (mg/kg)	ASU L 00.00-135 (ICP-MS)	< 0.06	< 0.06	< 0.06
Nickel (mg/kg)	ASU L 00.00-135 (ICP-MS)	0.12	0.1	0.09
Zinc (mg/kg)	ASU L 00.00-135 (ICP-MS)	4.7	3.5	15.4
Cobalt (mg/kg)	PV-347 ICP-MS	< 0.04	0.06	0.04

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

H. STABILITY

1. Genetic Stability of the Production Strain

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

2. Stability of 3-Fucosyllactose

The shelf-life of 3-FL is supported by two stability studies conducted on a mixture containing 2'-fucosyllactose (2'-FL), 3-FL, lacto-N-tetraose (LNT), 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL). The mixture contained approximately 14% 3-FL by dry weight after production and was stored in high density polyethylene bottles under ambient (25°C and 60% relative humidity) and accelerated (40°C and 75% relative humidity) conditions for 52 and 26 weeks, respectively. 3-FL and moisture content were monitored over time using the same methods that are used for batch qualification.

Although there appeared to analytical variability, 3-FL content remained relatively unchanged over the course of the 52-week testing period. Moisture content increased from 5.7% to 7.8 (Table 5).

Under accelerated conditions, 3-FL decreased and moisture increased over the course of the study (Table 6).

Additionally, because stability studies on other neutral HMOs, such as 2'-FL, LNnT, and LNT, show that they are stable for at least 3 years under ambient conditions and 1.5 years under accelerated conditions (EFSA NDA Panel, 2019; GRN 571; GRN 547; GRN 659; GRN 833), significant changes in the stability of 3-FL are not expected. Thus, together these results support a shelf-life for 3-FL of 2 years from the date of production when stored under ambient conditions.

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

Batch 4011-1004303107		Moisture		3-FL Content	
		%	% of baseline	% DW	% of baseline
Interval	Baseline	5.7	100.0	14.61	100
	Week 1	5.2	91.9	14.15	96.8
	Week 4	6.2	109.2	14.50	99.2
	Week 8	6.1	108.3	14.80	101.3
	Week 13	6.1	107.2	14.45	98.9
	Week 26	6.9	121.7	12.60	86.2
	Week 39	7.3	129.3	14.60	99.9
	Week 52	7.8	137.0	13.45	92.0

Abbreviations: DW, dry weight; 3-FL, 3-fucosyllactose; NA, not applicable.

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

Batch 4011-1004303107		Moisture		3-FL Content	
		%	% of baseline	% DW	% of baseline
Interval	Baseline	5.7	100.0	14.61	100
	Week 1	5.8	101.4	14.30	97.8
	Week 4	6.6	117.1	14.35	98.2
	Week 8	7.3	129.1	14.70	100.6
	Week 13	8.7	153.6	14.65	100.2
	Week 26	9.9	174.6	11.55	79.0

Abbreviations: DW, dry weight; 3-FL, 3-fucosyllactose; NA, not applicable.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

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

B. HISTORY OF EXPOSURE

3-Fucosyllactose is a naturally occurring oligosaccharide in human milk. It is also found to a lesser extent the milk of cows (Aldredge et al., 2013). Thus, humans have been exposed to 3-FL either through the ingestion of milk from humans or other mammals.

The concentration of 3-FL in human breast milk has been quantitated in 27 studies with greater than 5 donors (Asakuma et al., 2008; Austin et al., 2016; Austin et al., 2019; Azad et al., 2018; Chaturvedi et al., 1997; Chaturvedi et al., 2001a; Coppa et al., 1999; Coppa et al., 2011; Erney et al., 2000; Gabrielli et al., 2011; Kunz et al., 2017; Leo et al., 2010; Larsson et al., 2019; Ma et al., 2018; Marx et al., 2014; McGuire et al, 2017; McJarrow et al., 2019; Paganini et al., 2019; Nakhla et al., 1999; Samuel et al., 2019; Sjogren et al., 2007; Smilowitz et al., 2013; Spevacek et al., 2015; Sumiyoshi et al., 2003; Thurl et al., 2010; Van Niekerk et al., 2014; Williams et al., 2017). The results of 10 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 17 additional studies is presented in Table 7. Although 3-FL levels in breast milk vary with secretor status, time postpartum, and geographical location/study population (reviewed in Bode et al., 2012), the available studies show that the concentration of 3-FL in breast milk ranges from 0-5.9 g/L, with means and medians ranging from 0 to 2.4 g/L and 0 to 1.1 g/L, respectively. Therefore, the background exposure to 3-FL from human milk serves as the safe range for the use of Jennewein's 3-FL in infant formula.

Table 7. Studies Determining the Concentration of 3-Fucosyllactose (3-FL) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Timepoint(s)	3-FL concentration
Austin et al., 2016	China	Total 450 donors (90 donors/timepoint)	Days 5-11, 12-30, months 1-2, 2-4, and 4-8 of lactation	Reported range: 0.02-5.9 g/L Highest mean: 1.3 ± 0.9 g/L (4-8 months) Highest median: 1.1 g/L (4-8 months) Lowest mean: 0.5 ± 0.6 g/L (days 5-11) Lowest median: 0.2 g/L (days 5-11)
Austin et al., 2019	Switzerland	27 donors with 33 preterm infants (approx. 25 samples/timepoint) 34 donors with 34 term infants (approx. 28 samples/timepoint)	Weekly for 8 weeks after delivery (preterm and term) then every 2 weeks until 16 weeks (preterm only)	Reported range: 0.14 – 3.8 g/L Highest mean: 1.6 ± 0.7 g/L (Term, week 2) Lowest mean: 0.7 ± 0.4 g/L (Preterm, weeks 14 and 16)
Azad et al., 2018	Canada	427 donors	3- 4 months postpartum	Reported range: 0.02 – 3.0 g/L Mean: 0.5 ± 0.3 g/L Median: 0.5 g/L
Chaturvedi et al., 1997	Mexico	50 donors	1-2 months postpartum	Mean: 1.7 ± 0.07 g/L *Only mean ± standard error were reported
Kunz et al., 2017	Spain	32 donors, 96 samples	Lactation days 1-7 (colostrum), 8-15 (transitional milk), and 16-30 (mature milk)	Highest median: 1.1 g/L (0 – 1.2 g/L) (Term, mature Milk) Lowest median: 0 g/L (0 – 1.1 g/L) (Preterm, transitional and mature milk) *Only median and interquartile ranges were reported
Larsson et al., 2019	Denmark	11 mothers with high weight infants 15 mothers with normal weight infants	5 and 9 months	Highest median: 0.3 g/L (0.3 – 0.4 g/L) (5 months; high weight group) Lowest median: 0.2 g/L (0.2 – 0.3 g/L) (9 months; high weight group)
Leo et al., 2010	Samoa	8 mothers	5-10 days and greater than 10 days postpartum	Highest mean: 2.4 ± 1.4 g/L (greater than 10 days post-partum) Lowest mean: 1.7 ± 0.8 g/L (5-10days post-partum) *Median and range was not reported

Table 7. Studies Determining the Concentration of 3-Fucosyllactose (3-FL) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Timepoint(s)	3-FL concentration
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	Chinese Mothers Highest mean: 1.5 ± 0.8 g/L (120 days post-partum) Lowest mean: 0.5 ± 0.5 g/L (14 days post-partum) Malaysian Mothers Highest mean: 1.1 ± 0.7 g/L (365 days post-partum) Lowest mean: 0.5 ± 0.4 g/L (2 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	Mothers milk Reported range: ~0 – 0.4 g/L Median (interquartile range): ~0.1 (0 – 0.2) g/L Donor milk Reported range: ~0 – 0.6 g/L Median and interquartile range: ~0.3 (0.2 – 0.4) g/L *values obtained from a graph
McGuire et al, 2017	Around the World	410 donors	2 weeks to 5 months post-partum	Highest mean: 0.2 ± 0.03 g/L (Sweden; n=24) Lowest mean: 0.05 ± 0.008 g/L (Gambia Rural; n=40)
McJarrow et al., 2019	United Arab Emerites	Transitional milk: 41 donors Mature milk: 40 donors	Days 5-15 post-partum (transitional milk) 6 months post-partum (mature milk)	Highest mean: 1.2 ± 0.1g/L (Mature milk) Lowest mean: 0.6 ± 0.9 g/L (Transitional milk) *Only means ± standard deviations were reported
Paganini et al., 2019	Kenya	80 donors	No specific timepoint	Median (interquartile range): 0.8 (0.4-1.6) 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: 0.011 – 5.7 g/L Highest mean: 1.2 ± 0.7 g/L (day 120 postpartum) Highest median: 1.0 g/L (day 120 postpartum) Lowest mean: 0.4 ± 0.5 g/L (day 2 postpartum) Lowest median: 0.2 g/L (day 2 postpartum)

Table 7. Studies Determining the Concentration of 3-Fucosyllactose (3-FL) in Human Breast Milk				
Study	**Location**	**Number of Subjects/Samples**	**Timepoint(s)**	**3-FL concentration**
Sjogren et al., 2007	Sweden	11 allergic 9 non-allergic women	2-4 days postpartum	Range: 0.0 – 0.3 g/L Highest median: 0.7 g/L (Non-allergic mothers) Lowest median: 0.1 g/L (allergic mothers *Means were not reported
Spevacek et al., 2015	United States	Mothers of 15 term and 13 preterm	Colostrum (1st week), transition (14 days postpartum), and mature milk (28 d postpartum)	Highest mean: 1.0 ± 0.7 g/L (preterm, mature milk) Lowest mean: 0.4 ± 0.5 g/L (term, colostrum) *Medians were not reported
Sumiyoshi et al., 2003	Japan	16 donors	4, 10, 30, and 100 days postpartum	Reported range: 0.0 – 1.5 g/L Highest mean: 0.5 ± 0.1 g/L (100 days postpartum) Lowest mean: 0.2 ± 0.07 g/L (4 days postpartum) *Medians were not reported
Thurl et al, 2017	Around the World	Systematic review including 21 previous studies (not all reported 3-FL)	Lactation days 0 to >100	Highest mean: 0.4 g/L (95% CI 0.3-0.6 g/L; n=122 term mothers/365 samples) Lowest mean: 0.3 g/L (95% CI 0.2-0.5 g/L; n=75 preterm mothers/230 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 = 0.099 ± 0.019 g/L *Only one mean ± standard error was reported

C. INTENDED USES

Jennewein intends to use 3-FL in term, cow's milk-based non-exempt infant formula at a level of 0.91 g/L infant formula as consumed, which will adequately accommodate variations in 3-FL levels that occur due to ethnicity, secretor and Lewis-blood group status, lactation period, and term vs preterm birth.

D. ESTIMATED DAILY INTAKE

Non-exempt, term infant formulas will contain 0.91 g/L 3-FL powder as consumed. Infant formulas in the US market provide approximately 670 kcal/L (20 kcal/fl. oz.) (Martinez and Ballew, 2011). Assuming infant formula is the sole source of nutrition, it is reconstituted at a caloric density of 670 kcal/L (141 g/L), and the caloric requirements of a one month-old and six month-old infants are 472 kcal/day and 645 kcal/day, respectively (Institute of Medicine (US) Panel on Macronutrients and Institute of Medicine (US) Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 2005), 1 and 6 month-old infants consume approximately 0.704 and 0.963 L formula/day, respectively. The addition of 0.9 g/L 3-FL in infant formula will therefore result in an intake of 3-FL of approximately 0.64 g/day (146 mg/kg/day) for a 1 month-old infant and 0.88 g/day (115 mg/kg/day) for a 6 month-old infant.

IV. SELF-LIMITING LEVELS OF USE

This part does not apply.

V. COMMON USE IN FOOD BEFORE 1958

This part does not apply.

VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The general recognition of safety of 3-FL under the specified conditions of use in non-exempt term infant formula is based on the following: the published studies that have quantitated the levels of 3-FL in human milk (see Section III.B); published toxicology studies of 3-FL; the analytical data demonstrating that the 3-FL produced by Jennewein is structurally identical to 3-FL from human milk; the published toxicology studies with Dupont Health and Nutrition's ingredient; the qualitative and quantitative similarities between the subject of this Notice and Dupont Health and Nutrition's ingredient used in the published toxicology studies; and the corroborating toxicology and tolerability studies conducted with HMO mixtures containing Jennewein-manufactured 3-FL.

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

3-FL is a naturally occurring oligosaccharide in human milk. Numerous published studies have examined the level of 3-FL in human milk, and the reported range is 0-5.9 g/L with means and medians ranging from 0 to 2.4 g/L and 0 to 1.1 g/L, respectively. Higher levels of 3-FL are seen in non-secretor women and with increasing lactation time. Thus, Jennewein's intended use of 0.91 g 3-FL/L infant formula is well within the established range of 3-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 3-FL will not pose risks to infants consuming formula containing 3-FL.

The safe use of Jennewein's 3-FL ingredient in infant formula is supported by a battery of published and unpublished genotoxicity, subchronic toxicity, and tolerability studies (Pitt et al., 2019; Parschat et al., 2020; unpublished neonatal piglet study). Because Jennewein's 3-FL is qualitatively comparable and quantitatively similar to the 3-FL that is manufactured by Dupont Health and Nutrition and tested by Pitt et al. (2019), the genotoxicity and subchronic toxicity studies published by Pitt et al. are the pivotal results that support safety of Jennewein's 3-FL. 3-Fucosyllactose is not genotoxic and has a NOAEL (no observed adverse effect level) of at least 10% of the diet, which was the highest addition level tested and equivalent to 5.98 and 7.27 g/kg bw/day for males and females, respectively (Pitt et al., 2019). Additional genotoxicity and subchronic toxicity studies conducted by Parschat et al. (2020) and unpublished neonatal piglet

study, which were conducted with a mixture of HMOs containing 3-FL manufactured by Jennewein, corroborate the results reported by Pitt et al. (2019).

Based on these data, there is reasonable certainty that the use of Jennewein's 3-FL per the intended use and use level is of no harm to consumers. Jennewein's 3-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 authoritative bodies around the world (GRN 484, 2014; GRN 546, 2015; GRN 547, 2014; GRN 571, 2015; GRN 650, 2016; GRN 659, 2016; GRN 735, 2018; GRN 749, 2018; GRN 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 3-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).

Importantly, because the 3-FL that is the subject of this GRAS Notification is structurally identical to the 3-FL found in breast milk and the resulting estimated daily intake of 3-FL from the intended uses falls within the range of 3-FL intake from breast milk (see Section III.B), there is reasonable certainty that the absorption, distribution, metabolism, and excretion of 3-FL ingested from the intended uses at the intended use levels will mimic that from breast milk.

C. TOXICOLOGY

The toxicology studies that support the use of 3-FL in infant formula include a battery of published genotoxicity and subchronic toxicity studies (Pitt et al., 2019; Parschat et al., 2020). Specifically, Pitt et al. (2019) evaluated the genotoxicity, and acute and subchronic toxicity of a product containing 94.6 % 3-FL manufactured by DuPont Nutrition and Health in an OECD-compliant bacterial reverse mutation assay, an OECD-compliant chromosomal aberration assay,

an OECD-compliant *in vitro* micronucleus assay, an OECD-compliant *in vivo* micronucleus assay, an acute oral toxicity study, and an OECD-compliant 90-day feeding toxicity study. Parschat et al. (2020) 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 in an OECD-compliant bacterial reverse mutation assay, an OECD-compliant *in vitro* micronucleus assay, a seven-day pilot dietary toxicity study and an OECD-compliant 90-day feeding study. Both the 3-FL ingredients used by Pitt et al. and Parschat et al. were manufactured by fermentation using genetically engineered strains of *E. coli*, contained similar amounts of 3-FL, and have comparable carbohydrate by-products and other impurities such as protein, ash, and moisture, which are controlled by product specifications (Table 8).

Table 8. Comparison of Jennewein's 3-Fucosyllactose Ingredient with the Ingredient Tested by Pitt et al., 2019

Parameter	Pitt et al., 2019	Jennewein's Specifications
3-Fucosyllactose	94.60%	≥ 90.0%
Lactose	1.50%	≤ 5%
Glucose	1.3% (combined)	≤ 3%
Galactose		≤ 3%
Fucose	1.20%	≤ 3%
Other Carbohydrates	1.40%	n/a
Protein	≤ 100 µg/g	≤ 100 µg/g
Ash	< 0.5%	≤ 1.0%
Moisture	1.90%	≤ 9.0%

*Based on data presented in Table 3
n/a: not reported; 3-FL: 3-fucosyllactose: NLT: not less than; NMT: not more than.

Because Jennewein's ingredient containing 3-FL is qualitatively comparable and quantitatively similar to the 3-FL ingredient manufactured by Dupont Nutrition and Health and tested by Pitt et al. (2019), Jennewein can rely on the results published by Pitt et al. for the safety of their 3-FL ingredient. Additionally, although they administered lower amounts of 3-FL in their 90-day feeding study on an mixture of HMOs, the results reported by Parschat et al. corroborate the findings reported by Pitt et al. Briefly, 3-FL is not genotoxic, not acutely toxic following a single administration of 5000 mg/kg bw, and does not result in toxicologically significant or treatment-related effects in growth, feed intake or efficiency, clinical observations, or clinical or anatomic pathology changes at 10% of the diet (equivalent to 5.98 and 7.27 g/kg bw/day in males and females, respectively). Similar effects were reported by Parschat et al., although the lower doses of 3-FL were administered in combination with 2'-FL, LNT, 3'-SL, and 6'-SL. Brief summaries of the studies and results published by Pitt et al. and Parschat et al. are provided below.

1. Genotoxicity

a. Studies on 3-Fucosyllactose as a Single Ingredient

i. Bacterial Reverse Mutation Assay

The mutagenic activity of 3-FL was conducted in an OECD-complaint bacterial reverse mutation assay using 333, 667, 1000, 3333, and 5000 µg 3-FL/plate, with *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and *Escherichia coli* strain WP2uvrA in the absence and presence of exogenous metabolic activation (S9) (Pitt et al., 2019). 2-Nitrofluorene, sodium azide, ICR-191 acridine, and 4-Nitroquinoline-oxide were used as positive controls in conditions without S9, while benzo-[a]-pyrene and 2-amino-anthracene were used as positive controls in conditions with S9. Precipitation was not observed at any of the 3-FL concentrations, all negative control values were within the laboratory historical control range, and the number of revertants in the positive controls were 3-fold greater that those in the negative control. Importantly, 3-FL did not significantly increase the number of revertants either in the presence or absence of metabolic activation compared to the negative control, indicating that 3-FL was not mutagenic under the conditions tested.

ii. Mammalian Micronucleus Test

The ability of 500, 1000, 2500, 3500, or 5000 µg 3-FL/mL to induce micronuclei in CHO cells was evaluated in the absence and presence of metabolic activation in an OECD-compliant *in vitro* micronucleus test (Pitt et al., 2019). The number of micronuclei induced by 3-FL was compared to the number induced by water, the negative control, and the number induced by mitomycin C and cyclophosphamide, the positive controls. Although there was a statistically significant increase ($p < 0.05$) in average micronuclei frequency at 3-FL concentrations ≥ 2500 µg/mL relative to the negative control in the 4 hour S9-activated test condition according to a William's statistical test, there were no significant increases in the percentage of micronuclei ($p > 0.05$) at any of 3-FL concentrations in the presence or absence of S9 according to a Dunnett's test. Additionally, the statistically significant increases in the average micronuclei frequency at 3-FL concentrations ≥ 2500 µg/mL were not dose-dependent and within the 95% confidence interval (CI) of the laboratory historical control data (HCD) ranges, suggesting that the result was equivocal.

To determine whether the statistically significant increase at 3-FL concentrations ≥ 2500 µg/mL was reproducible, a confirmatory assay was conducted using 500, 1000, 2500, 3500, and 5000 µg 3-FL/mL with metabolic activation. A statistically significant ($p<0.05$) increase in

average micronuclei frequency was again observed at 3-FL concentrations ≥ 1000 µg/mL according to the William's, but not the Dunnett's test. However, consistent with the first assay, the statistically significant increases in average micronuclei frequency were not dose-dependent and within the 95% CI of the laboratory HCD range.

Because the cell cycle length during exposure was within acceptable limits (1.5–2.0 normal cell cycles), positive control substances demonstrated statistically significant ($p<0.05$) increases in micronucleus frequency compared with the concurrent negative control, all controls were within the laboratory HCD range, the study was considered valid. However, due to the reproducibility of the statistical trend in the S9-activated test system, the authors concluded that the study was equivocal, and neither positive nor negative in agreement with OECD guidelines.

iii. *In Vivo* Mammalian Erythrocyte Micronucleus Test

To determine whether 3-FL can induce micronuclei in mouse peripheral blood erythrocytes, groups of at least five male and female ICR mice were administered 3-FL at doses of 500, 1000, or 2000 mg/kg bw by oral gavage (Pitt et al., 2019) in an OECD-compliant *in vivo* micronucleus test. Mice receiving water and 30 mg/kg bw cyclophosphamide were run concurrently as negative and positive controls, respectively. Post-dosing peripheral blood samples were collected at 48 and 72 hours for the 3-FL and vehicle control groups, and at 48 hours for the positive control group. Significant increases ($p < 0.05$, Dunnett's test) in micronucleated reticulocyte frequency were observed in the positive control group and the results from the positive and negative control groups were within the laboratory historical control (HCD) range, confirming the validity of the study. Additionally, in the 500 mg/kg bw-treated male and female mice, plasma 3-FL concentrations were 572 and 681 ng/mL, respectively, confirming exposure. Although there was a significant decrease in reticulocyte frequency ($p<0.05$, Dunnet's test) at 48 hours post-dosing and a significantly decreasing trend in reticulocyte frequency ($p<0.05$, William's test) in male mice administered 2000 mg/kg bw 3-FL at 48 and 72 hours post-dosing, no statistically significant increases in micronucleated reticulocyte frequency in peripheral blood samples were noted at any of the 3-FL doses or time points tested. Thus, 3-FL was not genotoxic under the tested conditions in this study.

iv. Mammalian Chromosomal Aberration Test

To determine whether or not 3-FL could induce chromosomal abnormalities, an OECD-compliant mammalian chromosomal aberration test was conducted in cultured human lymphocytes using 3-FL concentrations of 1250, 2500, and 5000 µg/mL in the absence and presence of exogenous metabolic activation (S9) (Pitt et al., 2019). Cells were incubated with

demecolcine (0.1 µg/mL) to induce mitotic arrest prior to expansion in 0.075 M hypotonic potassium chloride and fixation with fresh methanol/glacial acetic acid (3:1 v/v). Mitomycin C was used as a positive control in −S9 conditions while cyclophosphamide was used as a positive control in +S9 conditions. There were no statistically significant increases in the frequency of structural chromosomal aberrations at any of the 3-FL concentrations. Thus, Pitt et al. concluded that 3-FL does not include chromosomal abnormalities at levels up to 5000 µg/mL.

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

i. Bacterial Reverse Mutation Test

To evaluate the mutagenicity of an HMO mixture containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein using fermentation, Parschat et al. (2020) conducted an OECD-complaint bacterial reverse mutation test. Five strains of *S. typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) were used in two independent experiments with and without metabolic activation. The first experiment was conducted as a plate incorporation test and the second as a preincubation test (Ames et al., 1973; Ames et al, 1975; Maron and Ames, 1983). Five, 10.0, 31.6, 100, 316 or 600 mg of the mixture containing 0.8, 1.6, 5.06, 16, 50.6, and 90 mg 3-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 90 mg 3-FL/plate) in either the plate incorporation or preincubation tests (Table 9). Parschat et al. concluded that the results indicate that the HMO mixture, and the 3-FL contained therein, was not mutagenic under the conditions tested.

Table 9. Bacterial Reverse Mutation Test Performed with an HMO Mixture Containing 16.0% 3-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 47.1% dry weight 2'-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 Test

To evaluate the clastsrogenicity and/or aneugenicity of an HMO mixture containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Jennewein using fermentation, Parschat et al. (2020) performed an OECD-compliant *in vitro* micronucleus test using human peripheral blood lymphocytes. Peripheral blood lymphocytes were obtained by venipuncture from young, healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation and exposed to 7.5, 15, 30, and 60 mg HMO mixture/mL medium (equivalent to 1.2, 2.4, 4.8, and 9.6 mg 3-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, both validating the tests. In contrast, no chromosomal damage was observed with the HMO mixture at any concentration or under any condition tested (Table 10). Thus, the HMO mixture was not genotoxic under the tested conditions at concentrations up to 60 mg/mL (9.6 mg/mL 3-FL).

Table 10. *In vitro* Micronucleus Test in Human Peripheral Blood Lymphocytes Exposed to an HMO Mixture Containing 16.0% 3-Fucosyllactose[b]

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]
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]
Negative control (water)	1.62	100	2000	4.0
7.5	1.59	97	2000	3.5
15	1.61	99	2000	2.0
30	1.57	93	2000	2.0
60	1.57	93	2000	2.0
Cyclophosphamide (20 µg/mL)	1.40	65	2000	26.5[a]

CBPI = Cytokinesis block proliferation index; RI = Replicative Index.
Values are means (n = 2)
[a] Significantly different from negative control ($p \leq 0.05$).
[b]The HMO mixture also contained 47.1% dry weight 2'-FL, 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 3-FL as a Single Ingredient

a. Acute Oral Toxicity Study

In an OECD-compliant acute oral toxicity study, the 3-FL product manufactured by Dupont Health and Nutrition was administered in SD rats by gavage at a limit dose of 5000 mg/kg bw (Pitt et al., 2019). All animals were fasted for 17.5 hr prior to dose administration. Feed was returned 3.5 hr post-dosing, and clinical signs of toxicity and body weights were recorded periodically over the following 14-days. All animals were then euthanized and examined macroscopically. There were no deaths or clinical signs of toxicity during the 14-day observation period and all animals gained weight. There were also no macroscopic observations during necropsy. Thus, 3-FL was not acutely toxic up to a dose of 5000 mg/kg bw.

b. 90-day Feeding Study on 3-FL

A 90-day subchronic oral toxicity study was conducted in SD rats according to OECD protocol 408 (Pitt et al., 2019). Animals (10/sex/group) received a control diet, diet containing 5% or 10% 3-FL ingredient manufactured by Dupont Health and Nutrition, or diet containing 10% FOS (reference group). All diets were consumed *ad libitum*.

The overall mean consumption of 3-FL at dietary concentrations of 5% and 10% was calculated to be 3038 and 5975 mg/kg bw/day, respectively, for males, and 3870 and 7270 mg/kg bw/day, respectively, for females. There were no deaths during the study period. There were no ophthalmological findings, clinical or physical observations, or effects on neurobehavioral parameters attributable to consumption of diets containing 3-FL. No statistically significant or biologically relevant differences in final body weight, overall body weight gain, feed consumption, or feed efficiency were observed. A significantly higher mean cell volume (MCV) and mean cell hemoglobin (MCH) were observed in the 5% 3-FL males ($p<0.05$), but this finding did not occur in the females of the 5% 3-FL treated-group or in the 10% 3-FL group and was not associated with changes in any other hematological parameters. There were no statistically significant differences in coagulation or quantitative urinalysis parameters. No statistically significant, biologically or toxicologically relevant differences in organ weights, macroscopic, or microscopic findings were observed among groups.

Mean serum concentrations of 3-FL in male rats ranged from 984-1520 ng/mL at 5% and 2080-2950 ng/mL at 10% of the diet, respectively. The serum concentrations in female rats were approximately 2-fold higher than male rats. The amounts recovered in urine (as the mol% of daily dietary intake) for male and female rats were 0.39 and 0.41% at 5% 3-FL and 0.35 and 0.36% at 10% 3-FL, respectively, indicating that low amounts of 3-FL were absorbed (< 1.0% of dietary intake) and that the systemic exposure to 3-FL was dose-dependent.

The NOAEL for 3-FL in this study was at least 10% of the diet, equivalent to 5.98 and 7.27 g/kg bw/day for males and females, respectively.

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

a. Seven-day Dietary Toxicity Study

In a seven-day pilot feeding toxicity study, female CD/Crl:CD rats (Charles River Laboratories, Sulzfeld, Germany) received either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Jennewein (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 3-FL was 1.6% 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 (16.0% 3-FL by dry weight, providing 3-FL as 1.6% of total diet) was chosen for the subsequent 13-week dietary toxicity study in rats.

b. Thirteen Week Dietary Toxicity Study

Parschat et al. (2020) fed either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Jennewein to rats for 90 days (n=10/sex/group) in an OECD 408-compliant 90-day dietary toxicity study. The HMO mixture contained 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL and 5.1% dry weight other carbohydrates, all of which were manufactured by fermentation. Thus, the overall dietary exposure to 3-FL was 1.6% 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). Detailed 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 3-FL of 0.8 to 1.10 g/kg bw/day in males and 1.0 to 1.27 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 (0.4-12.81 x 10^3 cells/µl) in both the control and HMO mix groups. Additionally, although the absolute number in one female receiving the HMO mix fell below the lower boundary of the historical control range (0.33 x 10^3 cells/µl), all neutrophil counts in the remaining males and females fell within the historical range. Thus, the statistically significant reduction in the absolute number of neutrophilic granulocytes observed in female rats administered HMO mix was deemed to be unrelated to test article administration.

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

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

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

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

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

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

Some statistically significant differences in absolute and relative organ weights were noted between control and the HMO mixture-treated groups (Table 9 and Table 10, respectively). Specifically, the absolute weight of the brains in HMO-treated male rats were lower ($p \leq 0.05$), the absolute weights of the right kidneys were lower in HMO-treated female rats ($p \leq 0.05$), and the relative weights of the left and right kidneys were lower in the HMO-treated female rats ($p \leq 0.05$). There were no significant differences in the absolute and relative weights of the other organs examined. Review of the individual animal data revealed that one female rat in the HMO-treated group had an absolute weight of the right kidney less than the lower boundary of the historical range for the laboratory. The left kidney of the same animal was also small relative to the other rats in the group (0.79 g versus a range of 0.92-1.12 g for the other female rats) and approached the lower boundary of the historical range (0.78-1.40 g). Together, these results indicated that the kidneys in this individual female were generally smaller than other rats in the HMO-treated group. None of the absolute or relative organ weight changes in the HMO-treated rats were associated with histopathologic changes. Therefore, because the brain and kidney changes were within the historical range for the laboratory, the kidney changes in the HMO group were exaggerated by a single animal with small kidneys, and the changes in the absolute and relative organ weights were not associated with adverse clinical chemistry effects or histopathologic changes, the significant differences in the absolute and relative organ weights in the HMO-treated group were deemed as normal biological variation.

Table 12. Significant Differences in Mean Brain and Kidney Weights

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

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

Table 13. Significant Differences in Mean Relative Kidney Weights

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

Abbreviations: N, number of animals; M, male; F, female; (l), left; (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: 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 16.0% 3-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 3-FL of 0.91 g/kg bw/day in males and 1.12 g/kg bw/day in females.

D. TOLERANCE STUDY IN NEONATAL PIGLETS

1. Introduction

To understand the safety and tolerance of a mixture containing Jennewein-manufactured HMOs (2'-FL, 3-FL, LNT, 3'-SL and 6'-SL; oligosaccharide blend) in a species other than the rat, a 21 day-neonatal piglet study was conducted. One animal died during the study due to a non-HMO-related bacterial infection. The mixture was well tolerated and did not produce any adverse effects on growth, development, hematology, clinical chemistry, organ weights, gross pathology or histopathology at levels up to 8 g/L. Although this study has not been published, the results corroborate the lack of adverse effects seen in the subchronic chronic oral toxicity study conducted by Pitt et al. (2019) with 3-FL and the subchronic rat dietary toxicity study conducted by Parschat et al. (2020) using a mixture of HMOs that contained 3-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 14):

Table 14. Experimental Design				
Group No.	Dose Concentration (g/L)	Dose Volume (mL/kg/day)	Number of Animals	
			Males	Females
1[a]	0[a]	500	6	6
2[b]	5.75	500	6	6
3[b]	8.0	500	6	6

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

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

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

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

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

Table 15. Clinical Pathology Sample Collection Plan					
Group No.[a]	Time Point(s)	Hematology	Coagulation	Chemistry	Urinalysis
1	Day 7 and Day 21	X	X	X	X[b]
2	Day 7 and Day 21	X	X	X	X[b]
3	Day 7 and Day 21	X	X	X	X[b]
Unscheduled Euthanasia	On occasion samples were collected from animals with an unscheduled euthanasia.				
Target Volume (mL)[c]:	NA	1 mL	1.8 mL	1.8 mL	All available
Method:	Urine samples were collected via cystocentesis at necropsy.				
Collection Site:	Anterior vena cava through the thoracic inlet				
Fasting Required:	Water was not available to the animals as the dosing formulations contain sufficient water for the piglets. Animals were not fasted prior to collection.				
Anticoagulant:	NA	K_2EDTA	Sodium Citrate	Serum Gel Separator	NA

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

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

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

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

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

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

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

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

3. Results

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

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

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

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

Clinical Observations: No test article-related clinical findings were observed at any of the Oligosaccharide Blend dose levels evaluated. The few clinical findings observed in the treated groups were either similar to those observed in concurrent controls and/or seen infrequently and/or considered common in animals of this species, strain, and age and unrelated to treatment (Table 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 17).

Table 17. Summary of Detailed Clinical Observations						
Observation Type: All Types	**Males**					
From Day 3 (Start Date) to 21 (Start Date)	**0 g/L**	**5.75 g/L**	**8.0 g/L**	**0 g/L**	**5.75 g/L**	
Total Number of Animals	6	6	6	6	6	6
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
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	-	-	-
Eyelid part/completely closed						
Number of Times Recorded	0	0	3	0	0	0
Number of Animals Affected	-	-	2	-	-	-
Abrasion(s)						

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

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

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

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

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

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

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

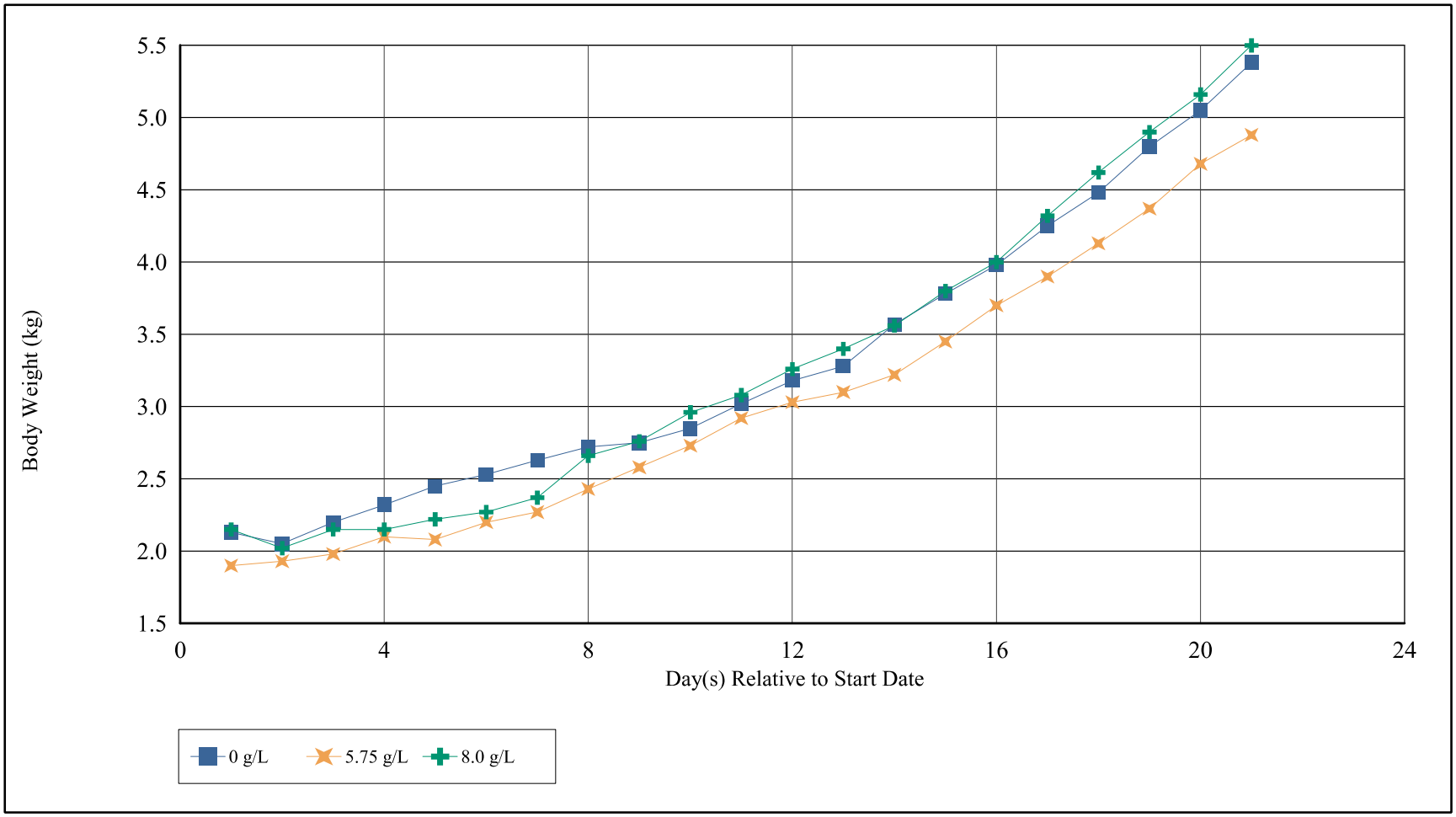


Figure 2a. Mean Body Weight Values (Male)

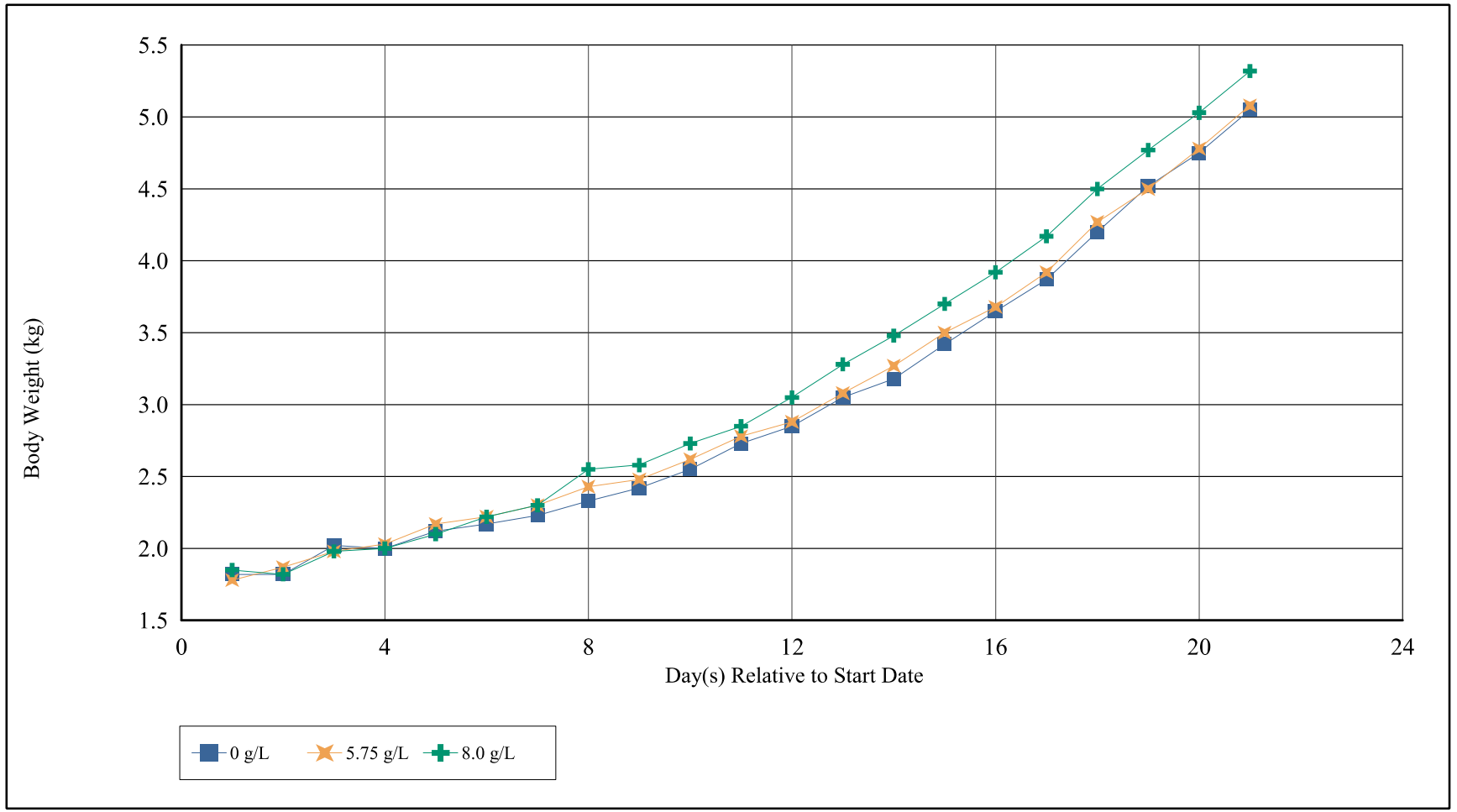


Figure 2b. Mean Body Weight Values (Female)

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

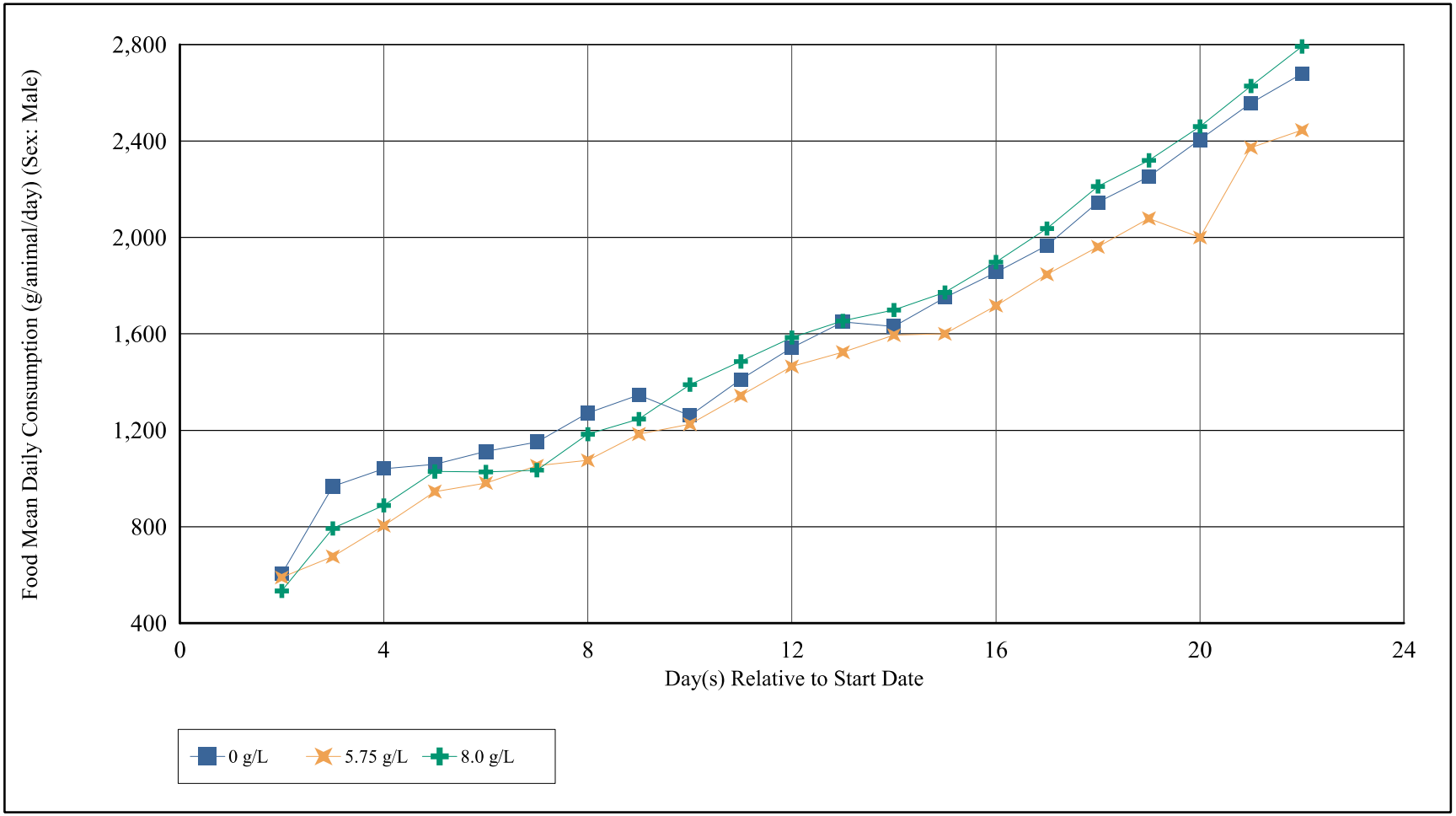


Figure 3a. Mean Feed Consumption Values (Male)

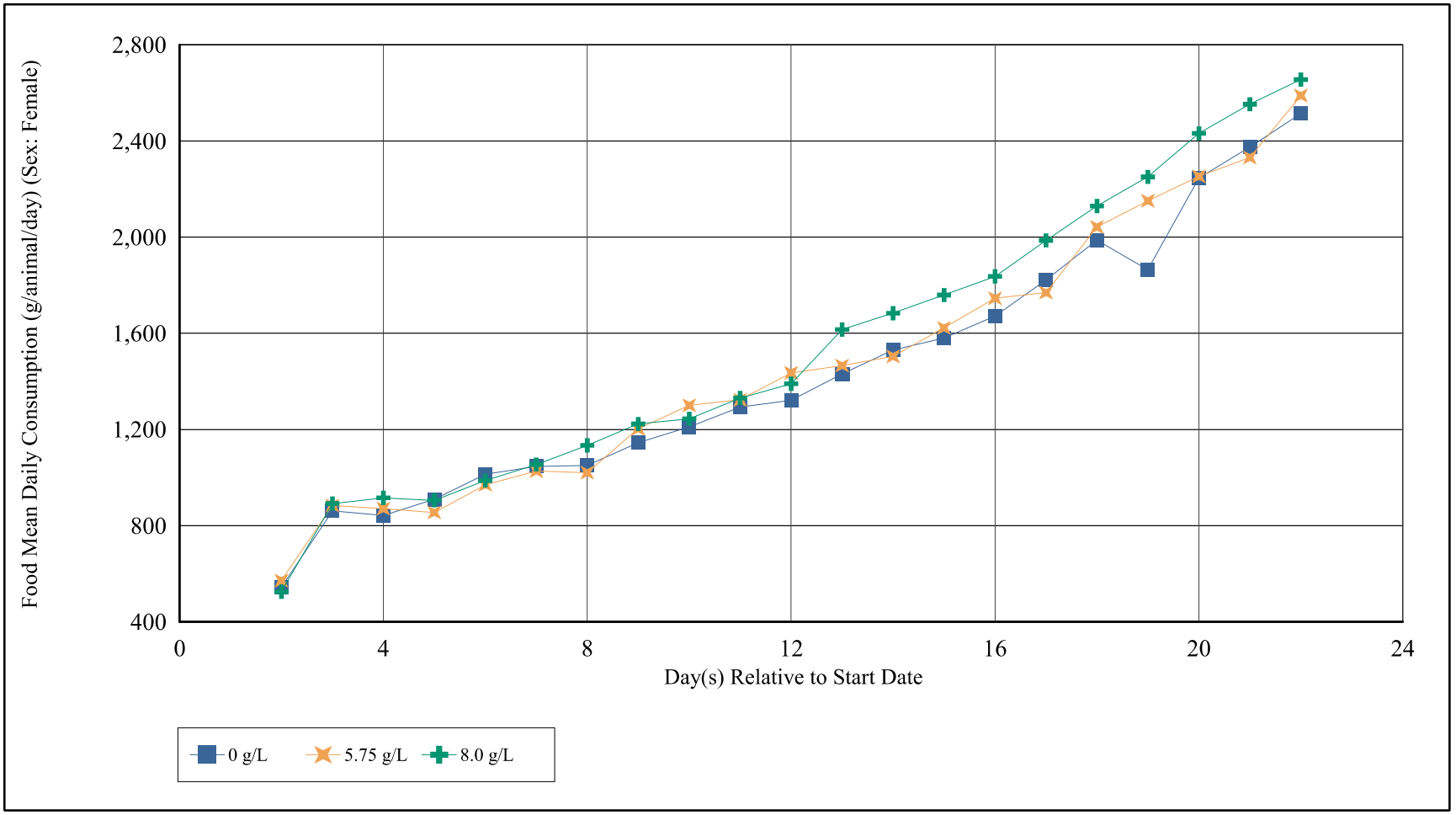


Figure 3b. Mean Feed Consumption Values (Female)

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

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

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

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

Clinical Pathology:

Hematology: Administration of Oligosaccharide Blend in the diet did not result in test article-related hematological changes (Table 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 22. Hematology (Mean ± St Dev (n))							
		Male			Female		
	Day	0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Leukocytes (10^3 cells/µL)	7 [g]	7.43 ± 1.846 (6)	6.65 ± 1.472 (6)	8.55 ± 4.437 (6)	8.94 ± 2.475 (6)	6.49 ± 1.387 (6)	7.67 ± 1.027 (6)
	21 [g]	10.13 ± 2.114 (6)	8.56 ± 2.488 (6)	8.53 ± 1.010 (5)	9.04 ± 1.907 (6)	8.87 ± 2.578 (6)	10.67 ± 4.078 (6)
Erythrocytes (10^6 cells/µL)	7 [g]	6.083 ± 0.5536 (6)	5.620 ± 0.4502 (6)	5.810 ± 1.0720 (6)	5.818 ± 0.8898 (6)	5.575 ± 0.5443 (6)	5.702 ± 0.6473 (6)
	21 [g]	5.985 ± 0.6187 (6)	5.973 ± 0.4604 (6)	5.572 ± 0.5601 (5)	5.537 ± 0.6020 (6)	5.817 ± 0.4597 (6)	5.847 ± 0.4652 (6)
Hemoglobin (g/dL)	7 [g]	11.32 ± 0.694 (6)	10.47 ± 1.033 (6)	11.22 ± 2.206 (6)	10.95 ± 1.390 (6)	10.38 ± 0.677 (6)	10.78 ± 1.082 (6)
	21 [g]	10.23 ± 0.753 (6)	9.78 ± 0.508 (6)	9.58 ± 0.976 (5)	9.62 ± 0.823 (6)	9.80 ± 0.626 (6)	9.97 ± 0.686 (6)
Hematocrit (%)	7 [g1]	37.88 ± 2.504 (6)	34.80 ± 3.239 (6)	37.93 ± 8.823 (6)	37.25 ± 4.678 (6)	35.27 ± 2.060 (6)	35.90 ± 3.994 (6)
	21 [g]	35.68 ± 3.301 (6)	34.42 ± 2.252 (6)	33.80 ± 3.648 (5)	33.43 ± 3.248 (6)	34.42 ± 2.460 (6)	34.95 ± 3.210 (6)
MCV (fL)	7 [g]	62.38 ± 2.121 (6)	61.90 ± 2.156 (6)	64.93 ± 3.579 (6)	64.27 ± 2.717 (6)	63.43 ± 2.601 (6)	63.12 ± 3.947 (6)
	21 [g]	59.68 ± 2.503 (6)	57.67 ± 1.388 (6)	60.64 ± 2.534 (5)	60.40 ± 1.287 (6)	59.17 ± 0.963 (6)	59.80 ± 3.517 (6)
MCH (pg)	7 [g]	18.65 ± 0.720 (6)	18.62 ± 0.649 (6)	19.30 ± 0.369 (6)	18.90 ± 0.800 (6)	18.68 ± 0.857 (6)	18.95 ± 1.017 (6)
	21 [g]	17.13 ± 0.747 (6)	16.40 ± 0.746 (6)	17.18 ± 0.512 (5)	17.42 ± 0.422 (6)	16.88 ± 0.417 (6)	17.07 ± 0.706 (6)
MCHC (g/dL)	7 [g]	29.88 ± 0.366 (6)	30.08 ± 0.694 (6)	29.78 ± 1.111 (6)	29.42 ± 0.436 (6)	29.42 ± 0.588 (6)	30.08 ± 0.556 (6)
	21 [g]	28.72 ± 0.981 (6)	28.43 ± 0.689 (6)	28.32 ± 0.526 (5)	28.80 ± 0.669 (6)	28.52 ± 0.504 (6)	28.55 ± 0.873 (6)
Platelets (10^3 cells/µL)	7 [g]	338.8 ± 129.95 (6)	376.3 ± 96.99 (6)	406.3 ± 79.71 (6)	338.0 ± 97.17 (6)	363.7 ± 97.07 (6)	375.8 ± 172.88 (6)
	21 [g]	525.0 ± 128.14 (6)	473.3 ± 155.96 (6)	518.2 ± 106.23 (5)	507.0 ± 152.52 (6)	534.2 ± 59.15 (6)	505.2 ± 88.16 (6)
Absolute Reticulocyte (10^3 cells/µL)	7 [g]	164.40 ± 26.996 (6)	202.83 ± 79.008 (6)	193.85 ± 98.450 (6)	191.13 ± 83.548 (6)	185.34 ± 49.619 (6)	199.70 ± 56.779 (6)
	21 [g]	505.10 ± 128.983 (6)	522.23 ± 144.895 (6)	447.01 ± 118.419 (5)	489.42 ± 64.458 (6)	579.73 ± 120.025 (6)	560.36 ± 136.182 (6)
Neutrophils (10^3 cells/µL)	7 [g1]	2.972 ± 0.6130 (6)	2.580 ± 0.5956 (6)	4.105 ± 3.2263 (6)	4.035 ± 2.0612 (6)	2.460 ± 0.7959 (6)	3.078 ± 0.9762 (6)
	21 [g]	3.465 ±1.2166 (6)	2.887 ± 0.9044 (6)	2.930 ± 0.8489 (5)	3.033 ± 1.2156 (6)	3.322 ± 1.7464 (6)	3.120 ± 1.3319 (6)
Lymphocytes (10^3 cells/µL)	7 [g]	3.953 ± 1.3391 (6)	3.613 ± 1.0854 (6)	3.907 ± 1.6667 (6)	4.348 ± 0.8825 (6)	3.590 ± 0.5723 (6)	4.055 ± 0.4197 (6)
	21 [g]	6.032 ± 1.5573 (6)	5.138 ± 1.7954 (6)	5.080 ± 1.3370 (5)	5.318 ± 1.0343 (6)	4.898 ± 0.7903 (6)	6.683 ± 3.7236 (6)

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

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

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

[g] – ANOVA & Dunnett (Log)

[g1] – ANOVA & Dunnett

[g2] – Kruskal-Wallis & Dunn

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

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

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

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

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

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

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

[g] – ANOVA & Dunnett

[g1] – Kruskal-Wallis & Dunn

[I] – n = Inappropriate for statistics

a = $p < 0.01$

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

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

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

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

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

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

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

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

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

A summary of the other absolute and relative organ weights is shown in Table 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 27. Absolute and Relative Organ Weights

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

Table 27. Absolute and Relative Organ Weights

Organ	Parameter	Male			Female		
		0 g/L	5.75 g/L	8 g/L	0 g/L	5.75 g/L	8 g/L
Large intes. [g] 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
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

Table 27. Absolute and Relative Organ Weights							
Organ	**Parameter**	**Male**			**Female**		
		0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
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
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

Table 27. Absolute and Relative Organ Weights							
Organ	**Parameter**	**Male**			**Female**		
		0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
Small 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
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

Table 27. Absolute and Relative Organ Weights							
Organ	**Parameter**	**Male**			**Female**		
		0 g/L	**5.75 g/L**	**8 g/L**	**0 g/L**	**5.75 g/L**	**8 g/L**
Abbreviations: BrWt - brain weight; BWt - body weight; duoden – duodenum; GB – gallbladder; gl – gland; intes/intest – intestine; w/ - with [g] – ANOVA & Dunnett [g1] – ANOVA & Dunnett (Log) [g2] – Kruskal-Wallis & Dunn A = $p < 0.05$							

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

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

4. Discussion

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

E. CLINICAL STUDIES

A literature search conducted through January 3, 2020 for "3-fucosyllactose" revealed no studies where 3-FL was administered to human subjects.

F. ALLERGENICITY

Allergens, by definition, are antigens that are recognized by IgE antibodies and provoke IgE-mediated hypersensitivity responses (Aalberse, 2000). Most allergens are proteins or glycoproteins (Radauer et al., 2008; Sicherer and Sampson, 1999), although there have been a limited number of reports of allergic reactions to carbohydrates (Franck et al., 2005; Chiang et al., 2012; Commins et al., 2009). Additionally, allergic reactions to human milk have not been reported. Importantly, genetically engineered strains of *E. coli* BL21(DE3) have been safely used in the production of food and pharmaceutical ingredients (see Section VI.A) and product specifications control the level of protein derived from *JBT-3FL* in the finished ingredient (see Section II.G). Moreover, the genes used to engineer *JBT-3FL* are not derived from major allergens and full-length FASTA alignments of amino acid sequences of the genes used to engineer *JBT-3FL* and version 19 of the AllergenOnline Database maintained by the University of Nebraska – Lincoln showed that cross-reactivity with known allergens (≥ 50% identity) is not expected (Table 28). Thus, although the protein specification does not completely eliminate the possibility that consumers of Jennewein's 3-FL-containing ingredient may be exposed to the protein residues derived from the production organism (specification of ≤ 0.01 % protein), allergic reactions resulting from the exposure to theoretically possible protein residues derived from *JBT-3FL* in the finished ingredient are not expected.

Table 28. Percent Identity of the Genetic Manipulations in *JBT-3FL* with Known Allergens

Function	Origin of the gene	% Identity*
Lactose permease	*E. coli* K12	27.4
UDP-galactose-4-epimerase	*E. coli* K12	30.7
Galactokinase	*E. coli* K12	None
Galactose mutarotase	*E. coli* K12	26.9
Galactosyltransferase	*E. coli* K12	24.0
Phosphomannomutase	*E. coli* K12	≤ 39.6
Mannose-1-phosphate guanosyltransferase	*E. coli* K12	None
GDP-fucose-4,6-dehydratase	*E. coli* K12	None
GDP-fucose synthase	*E. coli* K12	≤ 23
α1,3-fucosyltransferase	*Bacteroides fragilis* NTCT9343	None
Antibiotic Resistance Genes		
Dihydrofolate reductase conferring resistance to trimethoprim	*Citrobacter freundii*	≤ 34%
Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	None

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

G. REGULATORY APPROVALS AROUND THE WORLD

3-Fucosyllactose is a novel ingredient in the United States. It is also the subject of a Novel Food application in the European Union (https://ec.europa.eu/food/safety/novel_food/authorisations/summary-applications-and-notifications_en; accessed on February 10, 2020), although opinions by the European Food Safety Authority (EFSA) and/or the European Commission have not been published.

VII. SUPPORTING DATA AND INFORMATION

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B. EXPERT PANEL STATEMENT

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of 3-Fucosyllactose (3-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 3-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 3-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 % 3-FL dry weight.

    a. 3-Fucosyllactose is a neutral, fucosylated oligosaccharide in human milk.

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

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

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

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

f. Fermentation by-products include fucose and lactose, which are known components of human milk; their presence in the finished ingredient is not of toxicological concern.

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

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

2. Human milk oligosaccharides, including 3-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 3-FL in breast milk ranges from 0 to 5.9 g/L, with means and medians ranging from 0 to 2.4 and 0 to 1.1 g/L, respectively.

4. Genotoxicology and subchronic toxicology studies published by Pitt et al. (2019) show that 3-FL is not genotoxic and has a NOAEL (no observed adverse effect level) of 10% of the diet in rats, which was the highest level addition level tested and equivalent to 5.98 and 7.27 g/kg bw/day for males and females.

5. The addition of 0.91 g/L 3-FL in infant formula will result in an intake of approximately 0.64 g/day (146 mg/kg/day) for a 1-month-old infant and 0.88 g/day (115 mg/kg/day) for a 6 month-old infant.

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

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

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

c. The qualitative comparability and quantitative similarity of the Jennewein 3-FL to the 3-FL ingredient tested by Pitt et al. (2019).

d. Corroborative genotoxicology and 90-day subchronic dietary toxicology studies conducted with a mixture of human milk oligosaccharides published by Parschat et al. (2020), which contained 16% (dry weight) of Jennewein's 3-FL);

e. A corroborative unpublished tolerance study in neonatal piglets conducted with a mixture of HMOs containing up to 0.8 g/L of Jennewein-manufactured 3-FL study that showed an HMO mixture containing 3-FL was well-tolerated and supported normal growth in neonatal piglets.

Therefore, 3-FL is safe and GRAS at the proposed level of addition to the intended infant formula. 3-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: [redacted]

Date: March 19, 2020

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

Signature: [redacted]

Date: March 19, 2020

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

Signature: [redacted]

Date: March 19, 2020

Claire Kruger, PhD, DABT
Scientific Advisor to the Panel

Signature: [redacted]

Date: March 19, 2020

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

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000925	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

3-Fucosyllactose (3-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)* _____

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

SECTION D – CONDITIONS OF USE

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

Jennewein intends to use 3-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 3-Fucosyllactose (3-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;
(name of notifier)

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.

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 12:42:11 -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 3FL GRAS Final to FDA.pdf	Submission
	References	Submission

Spherix Consulting Group

October 21, 2020

Ellen Anderson
Consumer Safety Officer
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 000925

Dear Ms. Anderson:

In response to your email dated October 2, 2020, please find our responses to your request for additional information below. Please note that although we believe that the published literature adequately supports an intended use level of 0.91 g 3-fucosyllactose/L of infant formula, Jennewein Biotechnologie has decided to reduce the intended use level in this Notice to 0.44 g/L in non-exempt, cow's milk-based infant formula for term infants to be consistent with mean level calculated and published by Thurl et al. (2017). We hope that our responses adequately address your requests for additional information. If you have any additional questions or require any additional clarifications, please do not hesitate to contact me at dconze@spherixgroup.com.

Sincerely,

(b) (4)

Dietrich B. Conze, Ph.D.
Managing Partner

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

FDA's questions regarding GRN 000925 are in italicized text and our responses are in plain text.

1. *On page 5, Jennewein provides the common name, CAS No., IUPAC name, molecular formula, and chemical structure for 3-FL.*

   a. *Jennewein provides the incorrect molecular formula for 3-FL. Please provide the correct molecular formula for 3-FL.*

      The correct molecular formula is $C_{18}H_{32}O_{15}$

   b. *We note that 3-FL is considered to be a branched oligosaccharide and, according to the IUPAC recommendations, terms designating branches should be enclosed in square brackets (https://doi.org/10.1016/S0065- 2318(08)60090-6). The IUPAC name provided in the notice corresponds to an unbranched oligosaccharide, i.e., 3'-fucosylllactose (3'-FL). Please confirm that the subject of the notice is 3-FL as described by the common name and CAS No. and depicted by the chemical structure in the notice. In addition, please provide the correct IUPAC name.*

      The subject of this Notice is 3-FL, as described by the common name and CAS No. The correct IUPAC name is 6-deoxy-alpha-L-galacto-hexopyranosyl-(1->3)-[beta-D-galacto-hexopyranosyl-(1->4)]-D-gluco-hexopyranose.

2. *On page 5, Jennewein states that the structure of 3-FL produced by fermentation was confirmed using LC-MS/MS, ^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC and is consistent with the structure of 3-fucosyllactose. Please provide a sample chromatogram and ^{1}H NMR spectrum of Jennewein's 3-FL, as well as a ^{1}H NMR spectrum of a 3-FL reference standard for comparison.*

   A representative total ion current (TIC) chromatogram of 3-FL from an LC-MS/MS with Multiple Reaction Monitoring (MRM) analysis and the signature CID fragments 89, 179.3, 205.2 m/z of Jennewein's 3-FL and the 3-FL reference standard are provided in Figure 1. The results show that Jennewein's 3-FL has the same fragmentation pattern as the 3-FL reference standard.

   Representative ^{1}H NMR spectrums and a comparison of the Jennewein 3-FL and the 3-FL reference standard are provided in Figure 2 and 3, respectively. The results show that Jennewein 3-FL has the same ^{1}H NMR spectrum as the 3-FL reference standard.

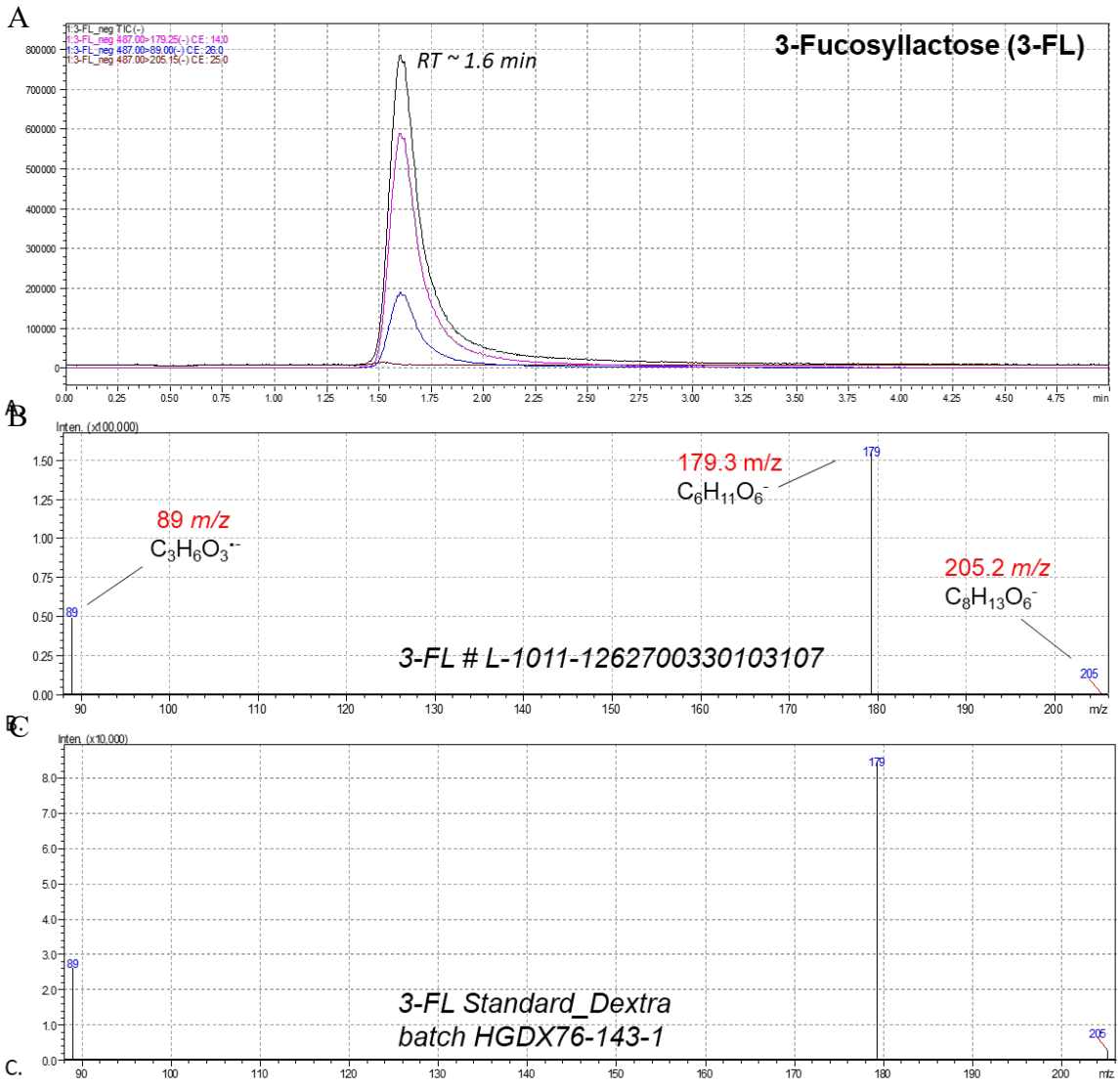
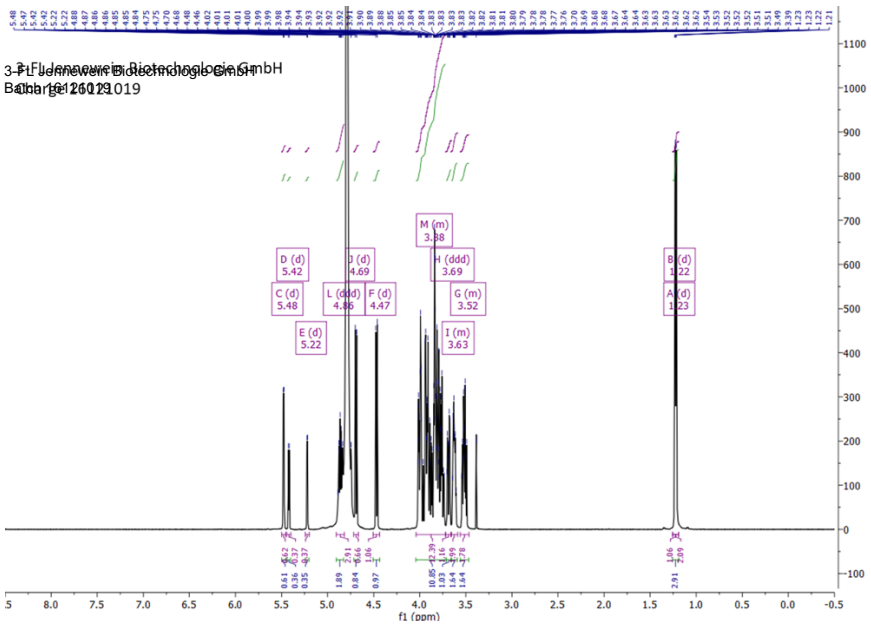


Figure 1. LC-MS/MS with Multiple Reaction Monitoring (MRM) analysis of 3-Fucosyllactose (3-FL). A. Total Ion Current (TIC) chromatogram of 3-FL eluting at ~ 1.6 min. B. Signature CID fragments 89, 179.3, 205.2 m/z of Jennewein Biotechnologie GmbH 3-FL (batch L-1011-1262700330103107). C. Signature CID fragments 89, 179.3, 205.2 m/z of Dextra Laboratories, Ltd. 3-FL reference standard (batch HGDX76-143-1).

A



B

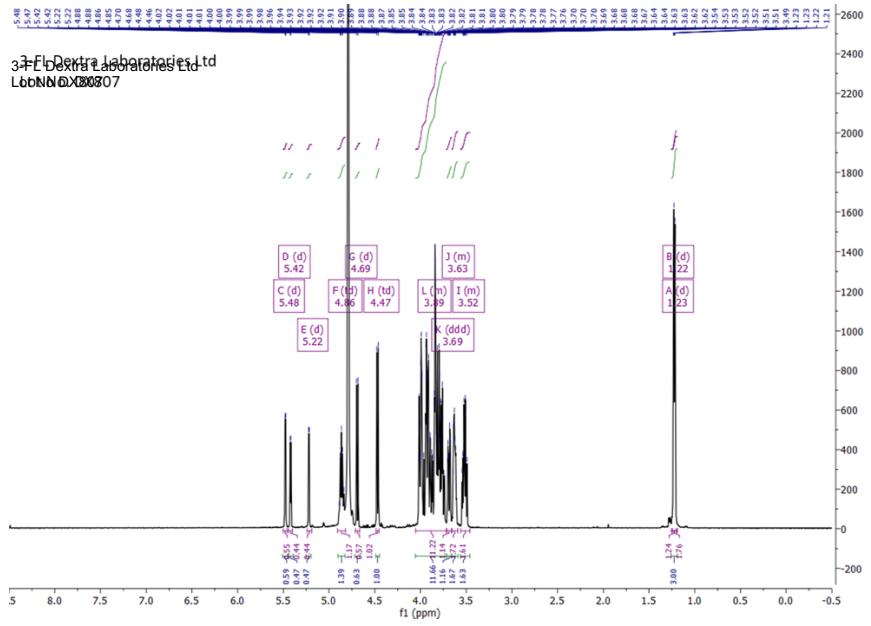


Figure 2. [1]H-NMR spectra of 3-Fucosyllactose (3-FL). A. [1]H-NMR spectrum of Jennewein Biotechnologie GmbH 3-FL (Batch 16121019) shown in the area from 8.5 to -0.5 ppm. [1]H NMR (500 MHz, D_2O) δ 5.48 (d, J = 4.0 Hz, 0.7H), 5.42 (d, J = 3.9 Hz, 0.3H), 5.22 (d, J = 3.8 Hz, 0.3H), 4.86 (td, J = 13.3, 6.7 Hz, 1H), 4.69 (d, J = 8.0 Hz, 0.7H), 4.47 (d, J = 7.8 Hz, 1H), 4.04 – 3.72 (m, 10H), 3.69 (ddd, J = 9.9, 3.4, 1.7 Hz, 1H), 3.66 – 3.59 (m, 2H), 3.56 – 3.47 (m, 2H), 1.23 (d, J = 6.6 Hz, 1H), 1.22 (d, J = 6.7 Hz, 2H). B. [1]H-NMR spectrum of Dextra Laboratories, Ltd. 3-FL reference standard (Lot No. DX807) shown in the area from 8.5 to -0.5 ppm. [1]H NMR (500 MHz, Deuterium Oxide) δ 5.48 (d, J = 4.0 Hz, 0.6H), 5.42 (d, J = 4.0 Hz, 0.4H), 5.22 (d, J = 3.8 Hz, 0.3H), 4.86 (dt, J = 6.9, 6.4 Hz, 1H), 4.69 (d, J = 8.0 Hz, 0.6H), 4.47 (td, J = 7.7 Hz, 1H), 4.05 – 3.71 (m, 10H), 3.69 (ddd, J = 9.9, 3.5, 1.7 Hz, 1H), 3.66 – 3.59 (m, 2H), 3.56 – 3.46 (m, 2H), 1.23 (d, J = 6.7 Hz, 1H), 1.22 (d, J = 6.6 Hz, 2H).

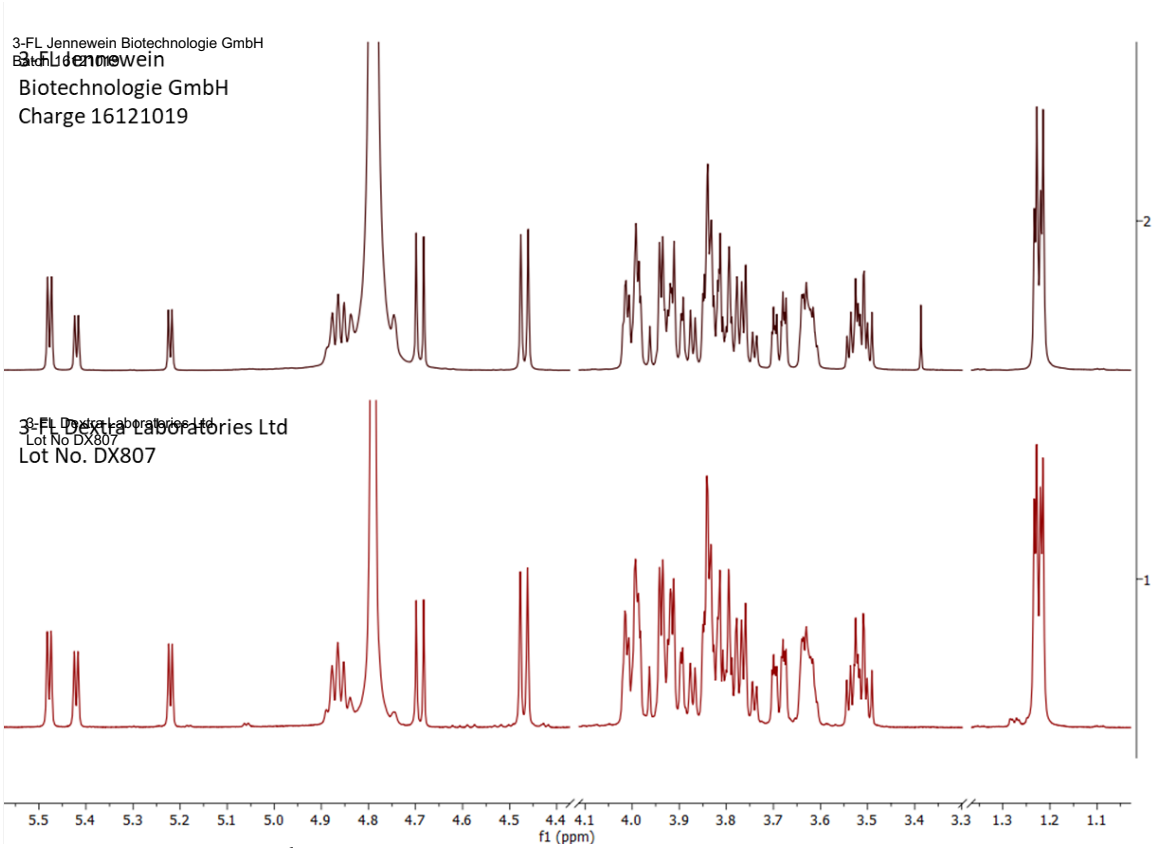


Figure 3. Comparison of [1]H-NMR-spectra of Jennewein Biotechnologie GmbH 3-Fucosyllactose (3-FL; Batch 16121019) and Dextra Laboratories, Ltd. 3-FL reference standard (Lot No. DX807).

3. *On page 5, Jennewein states that 3-FL contains residual impurities, i.e., lactose and other carbohydrate by-products. Please clarify whether the term "other carbohydrate by-products" refers to glucose, galactose, and fucose only or also includes other carbohydrates that can potentially be present in 3-FL. If carbohydrates other than glucose, galactose, and fucose are present, please provide information regarding their chemical identities and the total amount expected to be present in 3-FL. In addition, please provide a sample chromatogram of 3-FL with the constituents identified.*

   Glucose, galactose, and fucose are the only carbohydrate by-products produced during the manufacturing of 3-FL. Therefore the "other carbohydrate by-products" refers to only glucose, galactose, and fucose. A representative chromatogram of the high pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis is below (Figure 4).

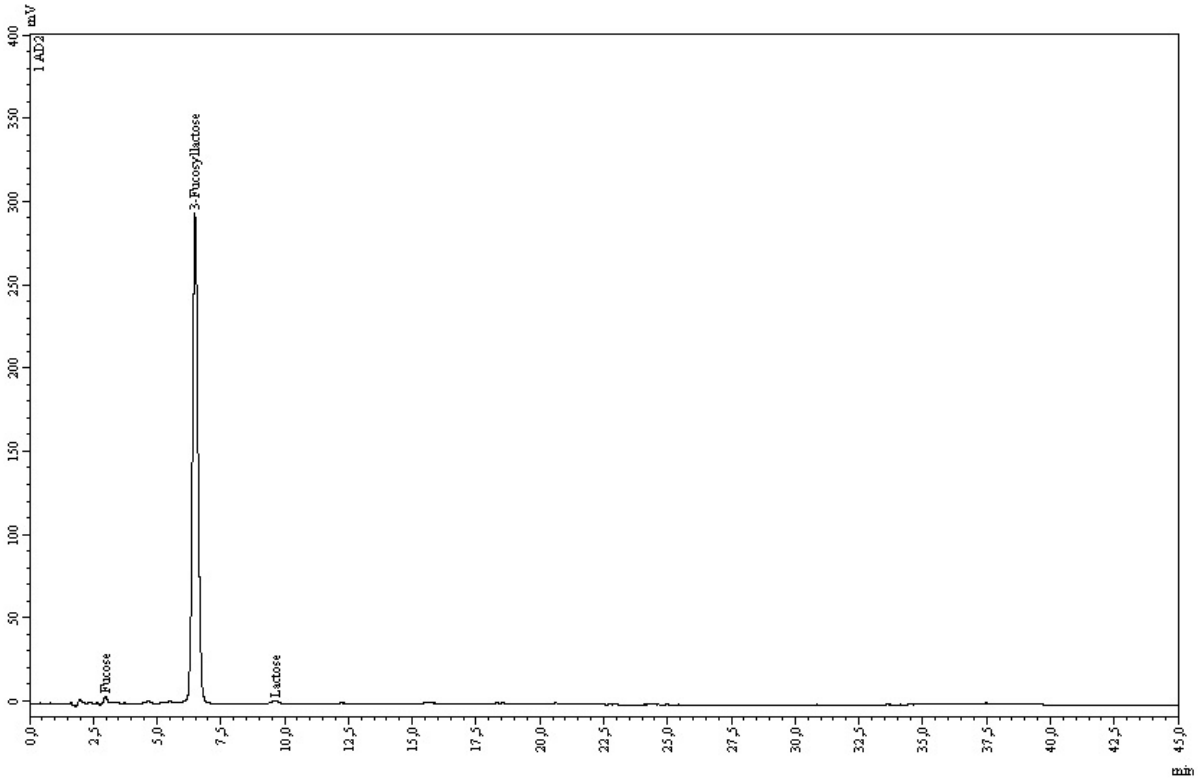


Figure 4. High pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) chromatogram of Jennewein Biotechnologie GmbH 3-Fucosyllactose (Batch 16121019). The peaks have been labelled for clarity.

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

The materials used in the production of the 3-FL that is the subject of this Notice include the production strain, medium components, and physical processing aids. The physical processing aids used in downstream processing where 3-FL is purified from the fermentation medium and concentrated, include activated carbon, ion exchange resins, and ultra- and nano-filtration membranes. All of these physical processing aids comply with the conditions of use specified in the U.S. Code of Federal Regulations. The medium components are essential for the growth of the production strain and 3-FL production. Importantly, they are actively consumed by the production strain, which is removed prior to downstream processing, are not direct food additives, and therefore considered to be "processing aids of a processing aid". If residues of these components remain in the 3-FL-containing medium after fermentation and removal of the production strain, they are then removed from the product using ion exchange chromatography and electrodialysis. Because similar medium components are used in the production of other human oligosaccharides that are GRAS and received "no questions" letters from the Agency (GRN 000571, GRN 000650, GRN 000735, GRN 000833, GRN 000852), all of the medium components used in the production of the 3-FL that is the subject of this Notice are considered GRAS for their intended use.

5. *On page 10, Jennewein states that the specification parameters are measured using compendial or internally validated methods. Please provide a statement indicating that all analytical methods are validated for their intended use.*

All analytical methods are validated for their intended use.

6. *On page 11 (Table 3), Jennewein lists the results of the batch analysis as "ND" for the heavy metals. Please confirm that "ND" in Table 3 indicates that the heavy metal in the respective sample is not detected at the limit of detection of the analytical method. If our understanding is not correct, please indicate what is meant by "ND" in Table 3.*

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

7. *On page 12, Jennewein provides batch analyses showing that cobalt was present at or above the limit of quantification in two of the three tested batches of 3-FL. According to information provided in GRN 571, which is incorporated by reference, cobalt chloride is used in the fermentation media. Under 21 CFR 189.20, food containing any added cobaltous salts is deemed adulterated. Therefore, cobalt should not be present in the final product. Please describe how Jennewein will produce 3-FL in compliance with 21 CFR 189.120 and provide analytical data from five nonconsecutive batches of 3-FL that demonstrate 3-FL can be produced without added cobalt remaining in the product.*

During downstream processing, the 3-FL-containing culture medium, which may contain residual amounts of the salts required for the growth of the production strain during fermentation, is subjected to ion exchange chromatography and electrodialysis. This

removes the residual elements from the finished product. To confirm that these processes remove cobalt, Jennewein quantitated the levels of cobalt in five additional batches of the finished ingredient (Table 1). Cobalt was below the limit of quantitation in all batches, indicating that the manufacturing process removes this medium element from the finished ingredient. Importantly, because of the regulatory status of cobalt (II) chloride and the potential for minute amounts to remain in the finished product, Jennewein will no longer use cobalt in their culture medium.

Table 1. Cobalt Analysis of 3-Fucosyllactose

Element[1]	Method	LOQ	Batch Number				
			11017049	11021029	16139019	26103010	26129040
Cobalt (mg/kg)	PV-347 (ICP-MS)	0.04 mg/kg	<0.04	<0.04	<0.04	<0.04	<0.04

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

8. *On page 19, Jennewein provides estimates of dietary exposure to 3-FL for one- and six-month-old infants based on the proposed use level of 3-FL and the energy requirements for infants reported by the Institute of Medicine (IOM, 2005).*

   a. *Jennewein does not explain why only two data points (i.e., infant ages of one month and six months) were considered in the dietary exposure assessment. We note that the estimates provided by Jennewein may not be representative for the range of infants from 0–12 months of age. Please provide estimates at the mean and 90th percentile for the range of infants from 0–6 months and 7–12 months of age. In addition, please explain how these estimates were derived and why these exposures should be considered representative for the infants of the specified ages.*

Mean and 90th percentile estimated 3-FL intakes for infant boys and girls ages birth to approximately 6 months were determined by dividing the two-week caloric intake on a body weight basis determined by Fomon (1993) by the typical caloric density of infant formula (670 kcal/L; Martinez and Ballew, 2011), resulting in the total infant formula intake/kg bw/day. The quotient was then multiplied by the intended use level of 0.44 g 3-FL/L, yielding the estimated intake of 3-FL from infant formula/kg bw/day. The resulting mean and 90th percentile estimated 3-FL intakes for boys and girls combined from birth to 6 months-old ranges from 0.06 to 0.08 g/kg bw/day and 0.07 to 0.09 g/kg bw/day, respectively. Additionally, as the boys and girls age, the mean and 90th percentile 3-FL intakes decrease from approximately 0.08 and 0.09 g/kg bw/day to 0.06 and 0.07 g/kg bw/day, respectively (Table 2). Importantly, these estimates assume that infants will consume the 3-FL-containing infant formula as the sole source of nutrition.

According to Grimes et al. (2015), who determined the dietary sources of total energy intake in infants and toddlers in the United States using the National Health and Nutrition Examination Survey 2005-2012 database, the actual total daily caloric intake from infant

formula decreases from 65 to 47% in infants from birth to 5.9 months-old to 6 to 11.9 months-old, respectively. Thus, the exposure to 3-FL from 7 to 12 months will be less than the exposure from 0 to 6 months. Therefore, considering these data and the fact that the calculations above assume that 3-FL-containing formulas will consumed as the sole source of nutrition, the exposure estimates for infants 0 to 6 months are conservative exposure estimates for infants 7 to12 months-old. Additionally, similar calculations have been used to support the safe use of other non-digestible carbohydrates, such as short-chain fructooligosaccharides in infant formula (GRN 000537), and the caloric intakes used in these calculations are corroborated by caloric intake data from the Feeding Infants and Toddlers Study 2016 (FITS 2016) (Bailey et al., 2018). In the FITS 2016 study, infants consumed a total of 663 and 894 kcal/day at the mean and the 90th percentile, which are similar to the caloric intakes determined by the Institute of Medicine (Institute of Medicine, 2005). Assuming a median body weight of 5.4 kg, which is the average median body weight of boys and girls combined at birth and 5 months (approximately 3.3 and 7.6 kg, respectively) per the infant growth charts issued by the Centers for Disease Control and Prevention (https://www.cdc.gov/growthcharts/who/boys_length_weight.htm; https://www.cdc.gov/growthcharts/who/girls_length_weight.htm), the resulting caloric intakes at the mean and the 90th percentile on body weight and daily basis, based on the FITS 2016 study data, are 118 and 165 kcal/kg/day, respectively.

Table 2. Estimated Daily Intake of 3-FL from Infant Formula

Interval (days)	Mean			90th percentile		
	kcal/kg/d[a]	Infant formula intake (L/kg)[b]	3-FL intake (g/kg/day)	kcal/kg/d	Infant formula intake (L/kg)	3-FL intake (g/kg/day)
Boys						
0-13	112.1	0.17	0.07	136.7	0.20	0.09
14-27	120.2	0.18	0.08	141.3	0.21	0.09
28-41	117.4	0.18	0.08	136.9	0.20	0.09
42-55	109.2	0.16	0.07	129	0.19	0.08
56-83	100.2	0.15	0.07	115.6	0.17	0.08
84-111	94.6	0.14	0.06	106.1	0.16	0.07
112-139	93.8	0.14	0.06	112.1	0.17	0.07
140-167	94.9	0.14	0.06	113.1	0.17	0.07
168-195	91.1	0.14	0.06	108.5	0.16	0.07
Girls						
0-13	110.7	0.17	0.07	135.5	0.20	0.09
14-27	117.6	0.18	0.08	138.9	0.21	0.09
28-41	114.2	0.17	0.07	136.8	0.20	0.09
42-55	108.2	0.16	0.07	127.4	0.19	0.08
56-83	100.2	0.15	0.07	114.4	0.17	0.08
84-111	95.2	0.14	0.06	106.8	0.16	0.07
112-139	97.5	0.15	0.06	113.1	0.17	0.07
140-167	93.6	0.14	0.06	113.3	0.17	0.07

168-195	90	0.13	0.06	107.9	0.16	0.07

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

b. Jennewein provides exposure estimates expressed in g/kg bw/d. We note that it appears that Jennewein used the median weights that approximate those reported by the IOM for boys. Please confirm that our understanding is correct. If not, please provide a reference for the body weights used in the exposure estimates. We further note that the body weights used to estimate dietary exposure to cobalt were different than those used to estimate dietary exposure to 3-FL. Please clarify the inconsistency in the body weights used in these exposure estimates.

Your understanding is correct. However, to address part "a" of Question 8, we have now calculated the mean and 90th percentile 3-FL estimated intakes for boys and girls ages 0 to 6 months-old using the caloric intake/kg body weight/day determined by Fomon et al. (1993). Importantly, by using the caloric intakes determined by Fomon et al. (1993), there is no longer an inconsistency in the 3-FL and cobalt exposure estimates.

c. We also note that exposures based on the median body weights for boys are lower (and thus less conservative) than those that would be based on the body weights for girls. Please provide exposure estimates based on the median weights for girls should Jennewein choose to use the body weights reported by IOM.

As stated above, we have now calculated the mean and 90th percentile 3-FL estimated intakes using the caloric intakes for boys and girls ages 0 to 5.9 months-old determined by Fomon et al. (1993), which are on a kcal/kg body weight/day basis. As shown in Table 2, boys and girls will be exposed to similar amounts of 3-FL through the ingestion of 3-FL-containing infant formula.

9. On page 19, Jennewein states that 3-FL is intended for use in cow's milk-based, non-exempt infant formula for term infants at a level of 0.91 g/L infant formula as consumed.

a. On page 16 in Table 7, Jennewein provides data on the reported concentrations of 3-FL in human breast milk. Jennewein cites Thurl et al., 2017, a systematic review that applied robust inclusion and exclusion criteria for the selection of 21 studies and reports a mean and 95% confidence interval for the concentration of 3-FL in human milk. In addition to Thurl et al., 2017, Jennewein provides data from 17 additional studies to provide a basis for the proposed use level of 0.91 g/L. We note, however, that several studies listed in Table 7 of the notice are either already included in the Thurl et al., 2017 analysis (see Table 2) or failed to meet the inclusion criteria and are excluded from the Thurl et al., 2017 analysis (see Tables S1 and S6):

Austin et al., 2016: Failed to meet inclusion criteria (Table S6)
Chaturvedi et al., 1997: Failed to meet inclusion criteria (Table S1)
Kunz et al., 2017: Failed to meet inclusion criteria (Table S6)
Leo et al., 2010: Already included in analysis (Table 2)

Marx et al., 2014: Failed to meet inclusion criteria (Table S1)
Sjögren et al., 2007: Failed to meet inclusion criteria (Table S1)
Spevacek et al., 2015: Already included in analysis (Table 2)
Sumiyoshi et al., 2003: Failed to meet inclusion criteria (Table S1)

On page 3 of the notice, Jennewein states, "The safety of exposure to Jennewein's 3-FL at its intended use level is supported by . . . (a) Published studies that quantitate the levels of 3-FL in human milk . . ." However, Jennewein's proposed use level for 3-FL is higher than the mean and the value for the upper 95% confidence interval reported by Thurl et al., 2017. Please provide a clear, detailed rationale that discusses the specific studies, study data, and the analysis (if any) used by Jennewein to justify the proposed use level of 0.91 g/L for 3-FL.

As discussed in Chapter III, Section B. History of Exposure, 3-FL is a naturally occurring oligosaccharide in human milk and its levels vary with secretor status, time postpartum, and geographical location. During our review of the studies included in Table 7 of the Notice and the systematic review conducted by Thurl et al. (2017), we found breast milk 3-FL levels ranging from 0 to 5.9 g/L, with means and medians ranging from 0 to 2.4 g/L and 0 to 1.1 g/L, respectively. Importantly, to be consistent with the mean level of 3-FL calculated and published by Thurl et al., Jennewein Biotechnologie will reduce the intended use level of their 3-FL ingredient in infant formula to 0.44 g/L. We would like to note, however, that Thurl et al. determined a mean 3-FL level in secretor mothers only. As discussed in part "b" of this question, secretor status only relates to a mother's ability to produce α−1,2-fucosylated HMOs, such as 2'-fucosyllactose and moreover, non-secretor mothers can produce 3-FL at levels higher than secretor mothers (Austin et al., 2019; Chaturvedi et al., 2001; Thurl et al., 2010; Gabrielli et al., 2011; Coppa et al., 2011). Thus, the mean calculated by Thurl et al. (2017) does not accurately represent the natural variability in the 3-FL levels that occur in the broader population, which includes non-secretor mothers.

To understand the relationship between the intended use level of 0.44 g/L and the levels of naturally occurring 3-FL in breast milk, we calculated a mean, median, central 68% confidence interval, 90th percentile, and distribution of the means reported in published studies in a non-parametric analysis using the following:

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

2. Means reported in the published studies included in the systematic review conducted by Thurl et al. (2017) (Asakuma et al., 2008; Bao et al., 2013; Chaturvedi et al., 2001; Coppa et al., 1999; Coppa et al., 2011; Smilowitz et al., 2013; Thurl et al., 2010; Van Niekerk et al., 2014; Gabrielli et al., 2011; Nakhla et al., 1999);

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

4. Means or medians reported in selected published studies excluded from the systematic review conducted by Thurl et al. (2017) (Bode et al., 2012; Kuhn et al., 2015; Newburg et al., 2004; Alderete et al., 2015; Austin et al., 2016; Kunz et al., 2017; Chaturvedi et al., 1997; Leo et al., 2010; Marx et al., 2014; Sumiyoshi et al., 2003);

5. Mean or medians from mothers that gave birth to preterm infants (Austin et al., 2019; Spevacek et al., 2015).

For the studies that were excluded from Thurl et al. (2017), we included Alderete et al. (2015), Austin et al. (2016), Chaturvedi et al. (1997), Kuhn et al. (2015), Bode et al. (2012), Marx et al. (2014), Sumiyoshi et al. (2003), and Spevacek et al. (2015) in our analysis because 3-FL levels can be higher in the breast milk of non-secretor mothers. Excluding these studies based on "unknown secretor status" would not allow for a comprehensive understanding of the naturally-occurring levels of 3-FL in breast milk. Newburg et al. (2004) was included because the milk samples were collected between 1 and 5 weeks of lactation. Excluding this study on basis of "lactation period was not fitting" would exclude valuable data that represents the natural variability in 3-FL levels during lactation. Thurl et al. (1996) was included because all means and data were treated equally in our calculations, regardless of the number of samples. Leo et al. (2010) and Spevacek et al. (2015) were included in our analysis because they were specifically excluded by Thurl et al. in their 3-FL analysis because the secretor status of the participants was unknown. Thus, excluding the studies conducted by Leo et al. (2010) and Spevacek et al. (2015) based on "unknown secretor status" would not allow for a comprehensive understanding of the naturally-occurring levels of 3-FL in breast milk. Bode et al. (2012), Marx et al. (2014), and Sjogren et al. (2007) all reported median levels and, in our analysis, if only medians were reported, then the medians were used in the analysis because they contain valuable data regarding the level of 3-FL in breast milk. The remaining studies excluded by Thurl et al. (2017) were not included in our analysis because they either did not quantify 3-FL in breast milk, did not provide the units for 3-FL that was quantified, or provided only relative amounts 3-FL in breast milk.

A total of two hundred and five data points were extracted from the included studies and the resulting mean, median, upper and lower limits of the central 68% confidence interval, and 90th percentile were calculated to be 0.68, 0.43, 1.34, 0.11, 1.50 g/L, respectively. The distribution of the means is presented in Figure 5. Based on our analysis, the intended use level and mean level calculated by Thurl et al. of 0.44 g/L falls below the mean of the reported means/medians in the included studies and therefore, secretor and non-secretor mothers.

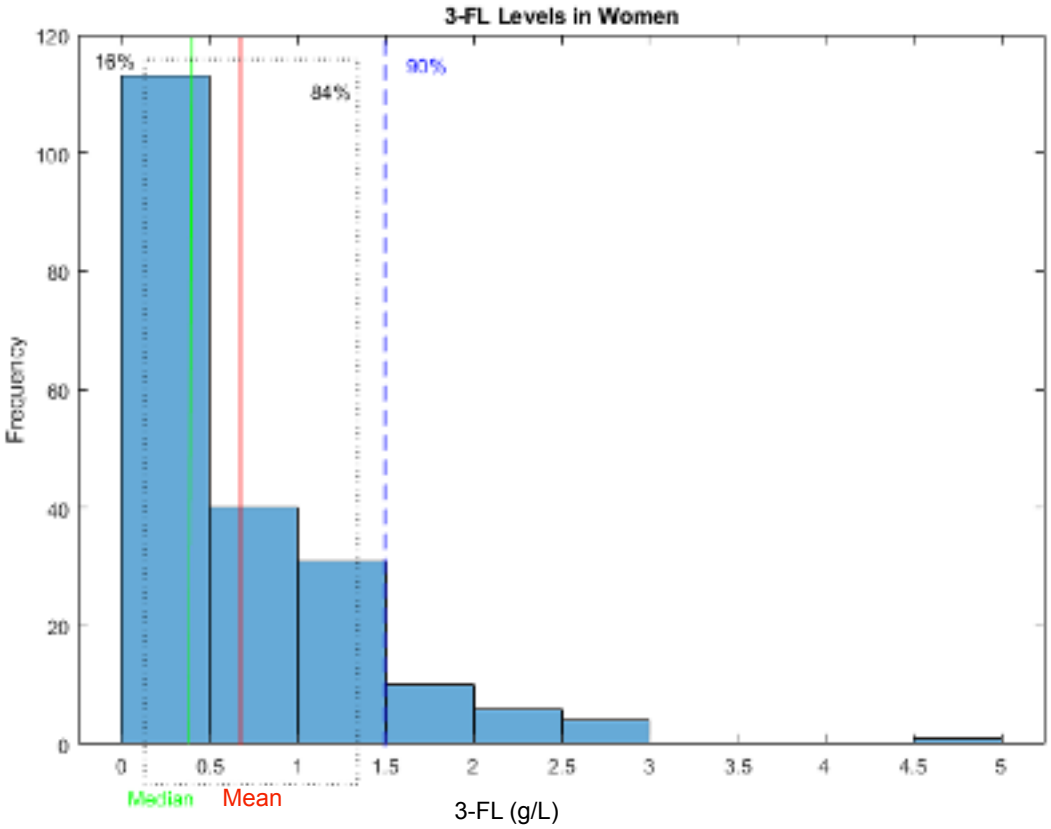


Figure 5. *The distribution, mean, median, the central 68% confidence interval, and 90th percentile of the mean/median levels of 3-FL in human milk. Two hundred and twenty-four means and medians were abstracted from the studies included in Table 7 of GRN 000925, reviewed by Thurl et al. (2017), as well as other publicly available studies. The histogram depicts the distribution of the 3-FL means/medians and the corresponding location of the mean (red line), median (green line), the upper and lower limits of the central 68% confidence interval (box with black dotted line), and the 90th percentile (blue dashed line).*

b. *Data on 3-FL in human milk as reported in Thurl et al., 2017 (Table S2-A) indicate that the levels of 3-FL increase with period of lactation. Additionally, the mean and the upper 95% confidence limit for 3-FL levels in human milk from lactation days 0 - 100 are less than the fixed use level of 0.91 g/L proposed by Jennewein. Thus, it is not clear how the levels of 3-FL found in human milk support safety of 3-FL in very young term infants, especially when their gastrointestinal system is considered underdeveloped compared to those of older infants. Given the lack of clinical studies demonstrating the tolerability in younger infants, please provide Jennewein's rationale that the proposed use level is tolerable to all infants ages 0-6 months, especially infants under 3 months of age.*

As summarized in GRN 000815 (see page 30 of GRN 000815), lactating mothers can be divided into four groups based on the expression of the fucosyltransferase-encoding genes FUT2 and FUT3, also known as the Secretor and Lewis genes, and the resulting HMOs that they produce. Group 1 expresses both FUT2 and FUT3, and synthesizes both

α−1,2-fucosylated and α−1,3/1,4-fucosylated HMOs. Group 2 expresses FUT3, but not FUT2, and synthesizes only α−1,3/1,4-fucosylated HMOs. Group 3 expresses FUT2, but not FUT3, and synthesizes only α−1,2-fucosylated HMOs. Group 4 does not express either FUT2 or FUT3 and cannot synthesize either α−1,2-fucosylated or α−1,3/1,4-fucosylated HMOs. Because Groups 1 and 3 express FUT2 and secrete high levels of 2-fucosyllactose (2'-FL), as well as other α−1,2-fucosylated HMOs, they have been deemed "secretor" mothers. Group 2 and 4, in contrast, have been deemed non-secretor mothers because they do not secrete 2'-FL or other α−1,2-fucosylated HMOs.

3-Fucosylactose is an α−1,3−fucosylated HMO that is synthesized by a variety of enzymes and although it is present in the milk of both secretor and non-secretor mothers, it can be produced a higher levels in non-secretor mothers that in secretor mothers (Austin et al., 2019; Chaturvedi et al., 2001; Thurl et al., 2010; Gabrielli et al., 2011; Coppa et al., 2011). Importantly, breast-feeding and human milk are the normative standards for infant feeding and nutrition (Section on Breastfeeding, 2012). Medical contraindications for breast feeding are rare and, when mother's milk is unavailable or in short-supply, donor breast milk is used because of the benefits associated with the ingestion of human milk (Committee on Nutrition, 2017), regardless of the secretor status of the donating mother or 3-FL content. In fact, the use of donor milk is based on a long and safe history of wet nursing, which was commonplace before the invention of infant formulas or the use of donor milk from milk banks (Moro, 2018). Taken together, this demonstrates that breast milk from mothers is fundamentally safe for ingestion by infants, including those 0 to 3 and 4 to 6 months old, regardless of their secretor status or HMOs levels. Additionally, 2'-FL and difucosyllactose, two structurally-related human milk oligosaccharides that differ only in the linkage between the fucose and lactose moieties and the number of fucose moieties, have been determined safe for use in infant formulas (GRN 000546; GRN 000571; GRN 000650; GRN 000735; GRN 000749; GRN 000815; GRN 000852; GRN 000897; Commission Implemented Regulation EU 2017/2470) and, moreover, 2'-FL-containing infant formulas are not contraindicated for use in infants born to non-secretor mothers, who would not normally be exposed to 2'-FL (https://similac.com/baby-formula/pro-advance, accessed on October 21, 2020; https://www.gerber.com/gerber-good-start-soothe-powder-formula; accessed on October 21, 2020).

Although the 3-FL levels in breast milk are highly variable and generally increase over the course of lactation, levels that exceed the intended use level of 0.44 g/L have been quantified in breast milk as early as four days postpartum (Alderete et al., 2015; Gabrielli et al., 2011). Other published studies have reported 3-FL levels ranging from 1 to 5 g/L in breast milk collected between 1 week to 3 months postpartum (Chaturvedi et al., 2001; Smilowitz et al., 2013; Austin et al., 2019; Ma et al., 2018; Samuel et al., 2019; Erney et al., 2000). Thus, the intended use level falls within the range that infants are exposed to through the ingestion of breast milk and is consistent with the mean level of 3-FL calculated in a systematic review of HMO concentrations in human milk (Thurl et al., 2017). Importantly, the safe use of 3-FL at the intended use level is also supported by a published, well-controlled sub-chronic rodent toxicology study conducted with 3-FL and

a neonatal piglet study, which is the surrogate for infant tolerability, conducted with a mixture of HMOs that included 3-FL (Parschat et al., 2020; Hanlon et al., in press). Additionally, 3-FL is also the subject of two Novel Food Petitions in the European Union for use in infant formula at a level of 1.2 g/L (https://ec.europa.eu/food/sites/food/files/safety/docs/novel-food_sum_ongoing-app_2019-1321.pdf; https://ec.europa.eu/food/sites/food/files/safety/docs/novel-food_sum_ongoing-app_2020-1620.pdf) and no studies have been published that demonstrate that the structurally-related HMO 2'-FL, which differs only in the linkage between the fucose and the lactose residues, is not tolerated by infants ages 0-6 months, infants under 3 months of age, or infants born to non-secretor mothers. Therefore, based on the publicly available data, there is reasonable certainty that the use of 3-FL in infant formula per the intend use level of 0.44 g/L will be tolerable to infants 0 to 3 and 4 to 6 months old.

10. *On pages 27-28, Jennewein discusses the results of the mammalian micronucleus test performed in CHO cells which was reported by Pitt et al., 2019 to be equivocal. Please provide a narrative that elaborates on why an equivocal result in this assay is not a safety concern and does not contradict your GRAS conclusion. Similar to the discussion in Pitt et al., 2019, such a narrative could include a discussion of the false positive rate of the assay, genotoxicity results of other structurally similar human milk oligosaccharides, the purity of the 3-FL ingredient., etc.*

Genotoxicity studies using cell lines, such as the mammalian micronucleus test performed in CHO cells by Pitt et al., 2019, can produce false-positive and therefore misleading results up to 53% of the time (Fowler et al., 2012). This lack of reliability is likely due to the perturbed metabolism, p53 function, and/or DNA-repair processes that allow the cell lines used in these studies to grow uninhibited in vitro. Because the results of the mammalian micronucleus test were equivocal when CHO cells were used, Pitt et al. (2019) conducted a follow-up micronucleus study using primary erythrocytes harvested from wild-type mice, which do not have perturbed metabolism, p53 function, or impaired DNA function. In the primary cells, there were no equivocal results and 3-FL was therefore determined to be not genotoxic. This is further corroborated by the lack of genotoxicity seen with other fermentation-produced neutral fucosylated human milk oligosaccharides such as 2'-FL and a mixture of 2'-FL and difucosyllactose, which differ only in the linkage between the lactose and fucose moieties and the number of fucose moieties (Phipps et al., 2018; Coulet et al., 2014; van Berlo et al., 2018). Additionally, the subject of this Notice lacks significant levels of impurities (see Table 8 of the Notice) that could affect the outcome of a genotoxicity study. Thus, based on the known error-rate of cell line-based genotoxicity studies, the results from the follow-up mammalian micronucleus test using primary mouse erythrocytes, the purity of the subject of this Notice, and the absence of genotoxicity reported for structurally similar fermentation-produced neutral fucosylated human milk oligosaccharides, it is reasonable to conclude that that 3-FL is not genotoxic and the equivocal results obtained by Pitt et al. (2019) using the CHO cells are not valid.

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

The strain has been deposited at DSMZ - German Collection of Microorganisms and Cell Cultures GmbH with the deposition number DSM 33491. The host strain from which JBT-3FL 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 3FL.

12. Please state whether E. coli BL21(DE3) strain "JBT-3FL" is non-toxigenic.

JBT-3FL is non-toxigenic.

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

Jennewein engineered JBT-3FL with genes that do not encode invasion factors, adhesion molecules and enterotoxins associated with virulence using site-specific homologous recombination or transposition. Therefore, JBT-3FL has the same virulence profile as *E. coli* BL21(DE3).

14. Jennewein states that E. coli BL21(DE3) is not expected to result in any safety concerns. Please state whether E. coli BL21(DE3) strain "JBT-3FL" is expected to result in any safety concerns.

JBT-3FL is not expected to result in any safety concerns.

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

E. coli BL21(DE3) is not able to transfer DNA to other organisms and during the engineering of JBT-3FL, Jennewein inserted genetic elements that do not confer the ability to transfer DNA to other organisms using site-specific homologous recombination or transposition. Therefore, JBT-3FL is not capable of DNA transfer to other organisms.

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

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

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From: Dietrich Conze
To: Anderson, Ellen
Cc: Claire Kruger; Kathy Brailer
Subject: Re: GRN 925 3-FL
Date: Thursday, November 05, 2020 4:21:45 PM

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

Regards and I apologize for the confusion.
Dietz

Dietrich Conze, PhD
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Fax: 301-230-2188
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> On Oct 23, 2020, at 5:37 PM, Anderson, Ellen <Ellen.Anderson@fda.hhs.gov> wrote:
>
> Thank you, Dietz. We will be in touch.
>
> Enjoy the weekend as well,
> Ellen
>
> ---
>
> **From:** Dietrich Conze <dconze@spherixgroup.com>
> **Sent:** Friday, October 23, 2020 4:41 PM
> **To:** Anderson, Ellen <Ellen.Anderson@fda.hhs.gov>
> **Cc:** Claire Kruger <ckruger@spherixgroup.com>; Kathy Brailer <kbrailer@spherixgroup.com>
> **Subject:** Re: GRN 925 3-FL
>
> Hi Ellen,
>
> Our responses to the Agency's questions are attached. If you have any follow-on questions, please let me know.
>
> Regards and hope you have a nice weekend.

From: Dietrich Conze
To: Anderson, Ellen
Cc: Claire Kruger; Kathy Brailer
Subject: Re: GRN 925 3-FL, clarification needed
Date: Monday, November 30, 2020 3:29:48 PM

Hi Ellen,

The statement that you are referring to is in Chapter 6, Section E. Clinical Studies and the purpose of the literature search that was conducted on January 3, 2020 was to identify clinical studies that evaluated the safety of ingesting 3-FL. The database that was used was PubMed and, as stated in the Notice, no clinical studies were identified. From a broader perspective, Chapter 6, the Narrative on the Conclusion of GRAS Status, includes all relevant published safety data on the intended use of 3-FL and is not limited in scope to human clinical studies only. Specifically, Chapter 6 includes reviews on the safety of the production organism, the absorption, distribution, metabolism, and excretion of HMOs, the toxicology studies conducted on 3-FL alone and mixtures containing 3-FL, the unpublished neonatal piglet study conducted on a mixture containing 3-FL, and the allergenicity of the 3-FL containing product.

Regards.
Dietz

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> On Nov 24, 2020, at 1:49 PM, Anderson, Ellen <Ellen.Anderson@fda.hhs.gov> wrote:
>
> Hello Dietz,
>
> We are just about finished with our evaluation of GRN 925, but have one more point of clarification to ask of you.
>
> On page 71 Jennewein states, “A literature search conducted through January 3, 2020 for “3-fucosyllactose” revealed no studies where 3-FL was administered to human subjects.” Please confirm that the literature search was conducted to encompass all relevant published safety data on the intended use of 3-FL and was not limited in scope to human clinical studies only. Please also state the databases used for the search.
>
> Thank you in advance for this information.

I hope you enjoy a peaceful Thanksgiving.

Sincerely,
Ellen

Ellen Anderson
Consumer Safety Officer

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