RAMBOLL

ENVIRONMENT
& HEALTH

Via Express Courier

Paulette Gaynor, PhD
GRAS Notification Program
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

Re: GRAS Notification for 2'-fucosyllactose: Exempt Infant Formula and Additional Uses

April 2, 2020

Dear Dr. Gaynor:

On behalf of Jennewein Biotechnologie, GmbH ("the Notifier"), Ramboll Environment & Health is pleased to submit a notification of the generally recognized as safe (GRAS) determination for the ingredient, 2'-fucosyllactose, manufactured using a genetically engineered *Escherichia coli* (*E. coli*) BL21 (DE3) strain as a processing aid, for certain exempt infant formula uses and additional uses as described in the dossier provided herein.

Sincerely,

Gavin Thompson, PhD
Principal Consultant

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cc: Stefan Jennewein, PhD

PN1690012704

GRAS ASSESSMENT OF JENNEWEIN 2'-FL FOR USE IN HYPOALLERGENIC INFANT FORMULAS AND TODDLER FORMULAS, AND PRETERM INFANT FORMULAS

Date
April 2020

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PART 1. SIGNED STATEMENTS AND CERTIFICATION

1.1 Name and Address of the Notifier

Stefan Jennewein, Ph.D.
Managing Director
Jennewein Biotechnologie GmbH
Maarweg 32
D-53619 Rheinbreitbach
Germany

1.2 Name of Notified Substance

The subject of this generally-recognized-as-safe (GRAS) notice (GRN) is 2'-fucosyllactose (2'-FL) manufactured using a genetically engineered *E. coli* BL21 (DE3) as a processing aid.

1.3 Background

Jennewein Biotechnologie GmbH (Jennewein) previously submitted a GRN (GRN 000571) to the U.S. Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety (OFAS) for the use of 2'-FL, manufactured using genetically engineered *Escherichia coli* (*E. coli*) BL21 (DE3) as a processing aid, in term infant formulas and toddler formulas. FDA issued a no-questions letter stating that "based on the information provided by Jennewein, as well as other information available to FDA, the agency has no questions at this time regarding Jennewein's conclusion that 2'-FL is GRAS under the intended conditions of use." (GRN 000571 Agency Response Letter, November 6, 2015). Further, FDA provided a no-questions letter (November 8, 2019) for Jennewein's supplemental submission to GRN 000571 regarding changes to the manufacturing process, specifically, strains used as processing aid (Appendix B). Herein, Jennewein is submitting this GRN of the assessment and GRAS status conclusion of additional food ingredient uses of 2'-FL.

1.4 Intended Conditions of Use of the Notified Substance

Jennewein intends to provide Jennewein 2'-FL as an ingredient for preterm infant formulas and hypoallergenic infant formulas in the United States. The use of infant formulas for preterm and hypoallergenic use begins at birth and toddler formulas for hypoallergenic use are formulas considered suitable for children from 12 through 35 months of age. The maximum target use of the ingredient in exempt infant formulas and toddler formulas is two grams (g) of 2'-FL per liter (L) of formula as consumed. The intended uses of the Jennewein 2'-FL product, and the typical and maximum concentrations are detailed in Table 1.

Table 1. Estimated Daily Intake of Jennewein 2'-FL from Infant Formula and Toddler Formula

Population[a]	n[b]	Percent users[c]	Formula Intakes Per User (L/d)		Jennewein 2'-FL Intakes Per User (g/d)	
			Mean	90th Percentile	Mean	90th Percentile
Infants, 0-5 mo	89	99.3%	0.9	1.2	1.9	2.5
Infants, 6-11 mo	111	93.9%	0.8	1.2	1.7	2.4
Toddlers, 12-35 mo	13	7.2%	0.4	0.6	0.8	1.2

Notes: [a] Breastfeeding infants and children were excluded from the sample population.

[b] Number of persons consuming infant formula during the study period.

[c] Weighted percent.

Concentrations of 2'-FL in formula based on maximum target addition level of 2'-FL to formula at 2.0 g/L. Formula intake data are based on National Health and Nutrition Examination Surveys (NHANES) 2015-2016 survey. Because this is a daily average, some participants who had day 1 but not day 2 data are included using a single day of consumption.

Abbreviations: 2'-FL = 2'-fucosyllactose; d = day; g = grams; L = liters; mo = months.

1.5 Statutory Basis for GRAS Determination

The use of Jennewein 2'-FL as an ingredient in exempt infant formulas (i.e. preterm infant formula and hypoallergenic infant formula) and hypoallergenic toddler formula at the levels described herein has been determined to be safe and GRAS, using scientific procedures, in accordance with the Federal Food, Drug and Cosmetic Act (FFDCA), Section 201(s) and Section 170.30 of Part 21 of the Code of Federal Regulations (21 CFR §170.30). The determination that the Jennewein 2'-FL food ingredient is safe, and GRAS, under the intended conditions of use, the safety of the proposed intake of Jennewein 2'-FL has been reviewed by experts qualified by scientific training and experience to evaluate the safety of substances directly added to food and is based on generally available and accepted information. These experts, independently and collectively, carefully and critically reviewed and evaluated the safety assessment as presented herein including the supporting data. The safety dossier incorporates publicly available information regarding the safety of 2'-FL including published reports of pivotal toxicological studies, unpublished supporting data from the Notifier, and estimates the potential human exposure to 2'-FL resulting from its intended use as an ingredient in exempt infant formulas for preterm infants and hypoallergenic use and hypoallergenic toddler formula. Jennewein and these experts concluded that the uses of 2'-FL described herein are safe and GRAS based on scientific procedures. The panel's report is presented in the Expert Panel Consensus Statement (Appendix A).

1.6 Exemption from Premarket Approval Requirements of the FFDCA

Jennewein has determined that the proposed food ingredient uses of 2'-FL, manufactured as described herein and meeting the specifications described herein, in exempt infant formula, i.e. preterm formulas and hypoallergenic formulas, and hypoallergenic toddler formula are exempt from the premarket approval requirements of the FFDCA because Jennewein determined such uses to be safe and GRAS. This determination was made in compliance with the Substances Generally Recognized as Safe regulation [21 CFR § 170.30, as published in

Jennewein concluded that the uses of 2'-FL described herein are safe and GRAS based on scientific procedures at the proposed levels of inclusion in exempt infant formulas and hypoallergenic toddler formula, and thus, these uses of 2'-FL are excluded from the definition of a food additive, are not subject to the premarket approval requirements of Section 201(s) of the FFDCA, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

1.7 Availability of Data and Information to FDA

Should FDA ask to see the data and information that are the basis for the conclusion of GRAS status of the food ingredient uses of Jennewein 2'-FL as described herein, Jennewein

i. agrees to make the data and information available to FDA; and

ii. agrees to the following procedures: upon FDA's request, Jennewein will allow FDA to review and copy the data and information as provided at 21 CFR §170.225(c)(7).

1.8 Freedom of Information Act (FOIA)

None of the data and information in this GRN is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

1.9 Certification

To the best of the knowledge of Jennewein Biotechnologie, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to Jennewein Biotechnologie and pertinent to the evaluation of the safety and GRAS status of the food ingredient uses of Jennewein 2'-FL described herein.

1.10 Name, Position, and Signature of Certifier

Based on an evaluation of relevant data laid out within this report, the notifier has determined that Jennewein 2'-FL is safe for its intended uses and GRAS under the terms of 21 CFR §170.30. We also have concluded that other "experts qualified by scientific training and experience to evaluate the safety of food and food ingredients" would agree with this determination.

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PART 2. IDENTITY, METHOD OF MANUFACTURE, AND SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

The identity, method of manufacture, and specifications of Jennewein 2'-FL were described previously in GRN 000571 and Supplement No. 1 to GRN 000571 for Jennewein 2'-Fucosyllactose: Modification to Manufacturing Process (hereafter referred to as "Supplement 1"). A description of the manufacturing process and characterization of the 2'-FL ingredient are presented in this Part 2.

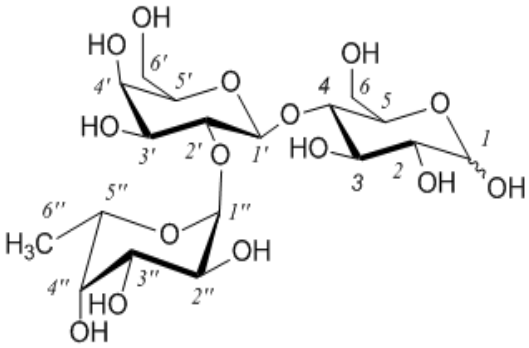
2.1 Identity

As previously described in GRN 000571, 2'-FL is a naturally-occurring oligosaccharide found in human breast milk and is one of the most abundant human milk oligosaccharides (HMOs). 2'-FL is an α-1→2 fucosylated lactose derivative represented by the empirical formula of $C_{18}H_{32}O_{15}$ and a molecular weight (MW) of 488.44 g/mole (mol). The full chemical name is α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranoside. This is often abbreviated as:

1. α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc; or
2. Fuc-α-1,2-Gal-β-1,4-Glc; or
3. 2'-FL

The substance is commonly known as 2'-fucosyllactose or 2'-fucosyl-D-lactose.

The Chemical Abstracts Service Registry Number (CASRN) for 2'-FL is 41263-94-9.



2'-Fucosyllactose
(α-L-Fuc-(1->2)-β-D-Gal-(1->4)-D-Glc)
(Molecular Formula = $C_{18}H_{32}O_{15}$)
Source: Jennewein Biotechnologie, GmbH

Figure 1. Chemical Structure of 2'-FL.

The Jennewein 2'-FL ingredient will be commercially available in two versions with identical sugar compositions: as a spray-dried lyophilized powder and as a liquid concentrate comprised of 45% 2'-FL content. "Jennewein 2'-FL" is herein used to distinguish Jennewein's commercially produced ingredient from naturally occurring 2'-FL.

2.2 Physical and Chemical Properties

The chemical and physical properties of Jennewein 2' FL are identified in Table 2.

Table 2. Chemical and Physical Properties of Jennewein 2'-Fucosyllactose Powder and Liquid Concentrate

Property	Powder Product	Concentrate Product	Method
Chemical			
Molecular weight	488.44 AMU	488.44 AMU	N/A
Solubility in water	min. 500 g/L (25 °C)	N/A	Visual
Stability (25 °C/65% humidity)	2 years from production date	6 months from production date	N/A
Physical			
Appearance (Form)	Fine, hygroscopic spray-dried powder	Liquid; clear solution	Visual
Appearance (Color)	White to ivory	Colorless to slightly yellow solution	Visual
Taste	Lactose-like	Lactose-like	N/A
Smell	Neutral	Neutral	N/A

Source: Jennewein Biotechnologie, GmbH

Abbreviations:
°C = degrees Celsius; AMU = atomic mass units; g = gram(s); L = liter(s); N/A = Not Available

2.3 Manufacturing Process

The manufacturing process of Jennewein 2'-FL for use in exempt infant formula and hypoallergenic formula is identical to that described in GRN 000571 and Supplement 1 which both received no questions letters from FDA for the use of 2'-FL in term infant and toddler formulas (Appendix B). A summary of the manufacturing process of Jennewein 2'-FL using *E. coli* BL21 (DE3) strains as a processing aid is included below. (A more detailed description is provided in GRN 000571, Section 2.2.2 p. 6 and in Supplement 1, Section 2.2 p. 3.)

The manufacturing process of Jennewein 2'-FL uses a genetically engineered E. coli BL21 (DE3) as a processing aid to produce the 2'-FL. To initiate the synthesis of 2'-FL by the E. coli BL21 (DE3) microbial cells via fermentation, the cells were genetically modified by introducing genes necessary to achieve the import of lactose and enhancing of GDP-fucose production. The synthesis of 2'-FL is followed by its export into the medium, and the subsequent degradation of excess lactose if necessary. Supplement 1 describes a slight modification to the E. coli BL21 (DE3) cells used in the manufacturing process, wherein the E. coli BL21 (DE3) cells do not have the ability to degrade excess lactose and a food-grade commercial lactase is added if excess lactose is present in the media.

2.4 Characterization of Production Organism

As previously described in GRN 000571 (Section 2.2.2 p. 6-7) and in Supplement 1 (Section 2.2 and 2.3 p. 3-5), the *E. coli* BL21 (DE3) strains used as a processing aid during the fermentation process in the manufacture of 2'-FL are genetically engineered strains of the commensal bacterium *E. coli* BL21 (DE3). The production strains were derived by genetic modification of the starting strain *E. coli* BL21 (DE3), which is a derivative of the *E. coli* B strain. The taxonomy of the species is described in Table 3**.** The *E. coli* B strain BL21 (DE3) is safe and well-characterised in terms of genetics and biochemistry, and benefits from the availability of genome engineering tools. *E. coli* BL21 (DE3) is often used for the heterologous expression of therapeutic proteins and used in the manufacture of a GRAS food ingredient (GRN 485).

Table 3. Taxonomic information on *E. coli* BL21 (DE3) strain

Taxonomy	Classification
Domain	Bacteria
Kingdom	Bacteria
Phylum	Proteobacteria
Class	Gamma-Proteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Escherichia
Species	*Escherichia coli*
Strain	*Escherichia coli* BL21 (DE3)

2.5 Product Specifications

To ensure that a consistent food-grade product is produced, Jennewein has established specifications for their 2'-FL product (GRN 000571, Section 2.3.2, p. 13-16). The specifications for Jennewein 2'-FL intended for use in exempt infant formula and hypoallergenic toddler formula are not different from those previously described and evaluated in GRN 000571 (Section 2.3.2, p. 16) and Supplement 1 (Section 2.4, p. 6). The chemical and microbiological specifications of the product are presented in Table 4 below (as presented in GRN 000571, Section 2.3.2, p. 16 and Supplement 1 Section 2.4 p. 6).

Table 4. Specifications of Jennewein 2'-Fucosyllactose Powder and Liquid Concentrate

Parameter	Powder Product Specification	Concentrate Product Specification	Method
Chemical			
Solids content	N/A	45% w/v (± 5% w/v) dry matter in water	Dry weight after freeze-drying
Water content	≤ 9.0%	N/A	Karl-Fischer titration
Protein content	≤ 100 µg/g	≤ 100 µg/g freeze-dried matter	Nanoquant (modified Bradford)
Total Ash	≤ 0.5%	≤ 0.5% freeze-dried matter	ASU L 06.00-4 (a)
Arsenic	≤ 0.2 mg/kg	≤ 0.2 mg/kg freeze-dried matter	ASU L 12.00-06 (a)
Cadmium	≤ 0.1 mg/kg	≤ 0.1 mg/kg freeze-dried matte	ASU L 00.00-19/3 (a)
Lead	≤ 0.02 mg/kg	≤ 0.02 mg/kg freeze-dried matter	ASU L 00.00-19/3 (a)
Mercury	≤ 0.5 mg/kg	≤ 0.5 mg/kg freeze-dried matter	ASU 00.00-19/4 (a)
Aflatoxin M_1	≤ 0.025 µg/kg	N/A	DIN EN ISO 14501
Endotoxins	≤ 300 EU/g	N/A	Ph. Eur. 2.6.14
GMO detection	negative	N/A	qPCR
Carbohydrate content			
2'-Fucosyllactose	≥ 90% (Area)	≥ 90% (Area)	HPAEC-PAD
Lactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
3-Fucosyllactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
Difucosyllactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
Fucosyl-Galactose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Glucose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Galactose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Fucose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Microbiology analysis			
Standard Plate Count	≤ 10000 cfu/g	≤ 5000 cfu/g	ISO 4833-2
Yeast and Mold	≤ 100 cfu/g	≤ 50 cfu/g	ISO 21527-2
Coliform / Enterobacteriaceae	absent in 11 g	absent in 22 ml	ISO 4832 / ISO 21528-2
Salmonella	absent in 100 g	absent in 200 ml	ISO 6579
Cronobacter sakazakii	absent in 100 g	absent in 200 ml	ISO/TS 22964

Source: Jennewein Biotechnologie, GmbH
Abbreviations:
ASU = Official collection of determination methods according to § 64 LFGB; cfu = colony-forming units; DIN EN ISO 14501 = German Institute for Standardization Milk and Milk Powder - Determination of aflatoxin M1 content - Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography; HPAEC-PAD = High-performance anion-exchange chromatography with pulsed amperometric detection; ISO = International Organization for Standardization; ISO/TS = International Organization for Standardization Technical Specifications; LFGB = German Code on food and feed; N/A = Not Available; Ph. Eur = European Pharmacopoeia; qPCR = quantitative polymerase chain reaction.

2.6 Batch Data

Characterization of multiple batches of Jennewein 2'-FL demonstrating compliance to the specifications in Table 4 were previously provided in GRN 000571 (Section 2.4 p. 16) and Supplement 1 (Section 2.4 p. 6).

2.7 Stability

Jennewein 2'-FL was demonstrated to be stable under both normal and accelerated storage conditions through 104 and 26 weeks, respectively (GRN 000571, p. 29-30).

2.8 Allergenic Potential and Absence of Protein

No allergenic material as listed in the Food Allergen Labeling and Consumer Protection Act of 2004 is used in the production of Jennewein 2'-FL other than lactose from cow's milk. The batch data and other analyses presented previously (GRN 000571 Section 2.4, p. 6 and Supplement 1 Section 2.6 p. 6) demonstrate that Jennewein 2'-FL is consistently devoid of proteins, bacteria or bacterial endotoxins, residual recombinant DNA, antibiotics, and chemical sensitizers including metals, or that they are well below levels of concern. Described below, Jennewein employed several analytical studies to demonstrate and confirm the absence of all potential allergenic proteins in the Jennewein 2'-FL. The potential allergenicity of Jennewein 2'-FL is extremely low and no sensitive populations have been identified or are anticipated.

2.8.1 Gel Separation Followed by Silver Stain

A silver stain SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) test with the ability to detect 0.2 to 0.6 ng of protein per band was performed using two lots of 2'-FL. 16 mg of 2'-FL per batch were prepared accordingly and run on an SDS polyacrylamide gel. The test was negative for protein in all five batches. Calculating back to the 2'-FL ingredient from the limit of detection of 0.2 ng of protein, this indicates that there is less than 10 µg/kg of protein present. Appendix C1 provides study results.

2.8.2 ELISA for Immunologically Active Casein and Whey

Five lots of Jennewein 2'-FL were tested according to an infant formula manufacturer's in-house Enzyme-Linked Immunosorbent Assay (ELISA) method for quantifying immunologically active casein and immunologically active whey. An ELISA is a standard biochemical assay used to detect the presence of, in this case, a protein using antibodies that will bind to the protein. The antibodies are linked to an enzyme which, when its substrate is added, allows the protein to be quantified.

Each sample was prepared at 0.22 g/L and ~1.1 g/L concentrations. No detectable casein or whey protein was detected in the five lots tested at 0.22 g/L. One of the five lots at 1.1 g/L concentration had a positive result for immunologically active casein protein of 25 mg of protein per kg of 2'-FL ingredient. This result, very near the method limit of quantitation of 9 mg/kg, may be an artifact of the competitive ELISA's tendency to overestimate values in non-matched matrices. Appendix C2 provides complete study results.

2.8.3 Size Exclusion Chromatography

Two lots of 2'-FL were tested for intact protein by size exclusion chromatography with ultraviolet (UV) detection at 205 mm. Size exclusion chromatography is a method wherein molecules in solution are separated by size as the solution is pulled through a chromatography column. No intact protein was detected in either of the two lots. The method detection limit in the test was 50 µg of intact protein per 1 g 2'-FL. Appendix C3 provides complete study results.

2.8.4 Absence of Protein: Summary and Interpretation of Analytical Results

Considered together, the analysis for protein using three different, complimentary analytical methods demonstrates an absence of protein in Jennewein 2'-FL. The most sensitive of the methods (silver stain, limit of detection 10 µg/kg) was unable to detect any protein in Jennewein 2'-FL. The silver stain method is a non-specific analysis that has the capability of detecting any protein present, as opposed to the ELISA method which is targeted to specific proteins (in this case milk proteins, either casein or whey). The other non-targeted method of protein analysis, size exclusion chromatography, also demonstrated an absence of any protein, albeit at a higher limit of detection (50 mg/kg). While the ELISA test for immunologically active casein had a positive result in one of the lots (25 mg/kg), very near the limit of quantitation for the method (9 mg/kg), there is good support that this is a spurious finding since it was very near the limit of quantitation for a method known to produce artifacts in non-matched matrices, the absence of detection in the size exclusion chromatography, and perhaps most importantly the absence of any protein detected in the silver stain method that has a limit of detection 1000-fold lower than the limit of quantitation of the ELISA method.

2.9 Intended Technical Effect

In accordance with 21 CFR 170.3(o)(20), the intended effect of adding ingredient to exempt and nonexempt infant formulas and toddler formulas is as a nutrient necessary for the body's nutritional and metabolic processes.

Further, 2'-FL as an HMO serves as a prebiotic for commensal gut bacteria which metabolize prebiotics into short-chain fatty acids used for energy by colonocytes, and to stimulate sodium and water absorption (Rodricks *et al*., 2007, Engfer *et al*., 2000). 2'-FL helps encourage growth of beneficial bacteria (e.g. bifidobacteria) in the infant's intestine (Engfer *et al*., 2000; Marcobal & Sonnenburg, 2012; Yu *et al*., 2013; Weichert *et al*., 2013; Morrow *et al*., 2004).

PART 3. DIETARY EXPOSURE

3.1 Background Intake Level

2'-FL is a naturally-occurring HMO found in mother's milk. The levels of 2'-FL in milk vary from individual to individual, mainly due to secretor status of the lactating mother, and the mean concentration of 2'-FL normally found in breast milk is between 0.7 g/L and 3.8 g/L (Goehring *et al*., 2014, McGuire *et al*., 2017). Part 5 of this dossier describes exposure to naturally-occurring 2'-FL in the context of the proposed uses.

Jennewein Biotechnologie provides 2'-FL to manufacturers of infant formula in the United States. Infant formula is defined by the FFDCA as "a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk" (FFDCA 201(z)). The maximum level of Jennewein 2'-FL in exempt infant formulas and hypoallergenic toddler formulas is 2 g 2'-FL per liter as consumed. The use level of 2 g/L corresponds to the use level determined to be safe in GRN 571 and is in the range of the concentration of 2'-FL normally found in breast milk.

3.2 Preterm Infant Formula

Jennewein intends to use Jennewein 2'-FL in exempt infant formulas for preterm infants in accordance with 21 CFR 107.3. The maximum target use of 2'-FL in exempt infant formula for preterm is the same as in nonexempt formula (GRN 000571 Section 4.2 p. 24 and Supplement 1 Section 3.2 p. 7): 2 g 2'-FL per L formula as consumed resulting in an estimated daily intake (EDI) of 2 g 2'-FL per day (assuming a consumption of 1 L/d).

3.3 Hypoallergenic Infant Formula

Jennewein also intends to use Jennewein 2'-FL in exempt infant formulas for hypoallergenic use in accordance with 21 CFR 107.3. The maximum target use of 2'-FL in exempt infant formula is the same as in nonexempt formula (GRN 000571 Section 4.2 p. 24 and Supplement 1 Section 3.2 p. 7): 2 g 2'-FL per L formula as consumed resulting in an EDI of 2 g 2'-FL per day (assuming a consumption of 1 L/d).

3.4 Hypoallergenic Toddler Formula

Jennewein also intends to use Jennewein 2'-FL in hypoallergenic toddler formula in accordance with 21 CFR 107.3. The maximum target use of 2'-FL in hypoallergenic toddler formula is the same as in toddler formula (GRN 000571 Section 4.2 p. 24 and Supplement 1 Section 3.2 p. 7): 2 g 2'-FL per L formula as consumed resulting in an EDI of 2 g 2'-FL per day (assuming a consumption of 1 L/d).

3.5 Estimated Intakes of 2'-FL from the Proposed Uses

Estimates of potential intakes of ingredient resulting from these intended uses were calculated using food consumption data reported in the United States Department of Health and Human Service's 2015-2016 National Health and Nutrition Examination Survey (NHANES). EDIs were calculated assuming all formulas for infants and toddlers contained 2 g 2'-FL per L formula (as consumed). The highest estimated mean intake of Jennewein 2'-FL occurs in infants of 0-5 months and is 1.8 g/d and the estimated intake at the 90th percentile is 2.5 g/d (Table 5). A small number of infants consume formulas after the first year of life, and formula intakes are lower than intakes by infants 0-5 or 6-11 months of age. The estimated mean and 90th percentile 2-day average intakes of 2'-FL by toddlers, the target consumers of toddler formulas, are 1.1 and 2.0 g/day, respectively. The estimates of

potential ingredient intake by infants were based on the survey population of non-breastfed infants. The youngest infants (i.e., prior to introduction of weaning foods) in the sample population, therefore, are consuming only infant formula. Some infants and toddlers, however, consume a combination of human milk and formula. Because the use level of 2 g/L is in the range of concentration of 2'-FL normally found in breast milk, the overall intake of 2'-FL by combination breast milk/formula fed infants and toddlers likely would be comparable to the intake of 2'-FL by infants receiving breast milk only.

Table 5. Estimated Daily Intake of Jennewein 2'-FL from Infant Formula and Toddler Formula

Population[a]	n[b]	Percent users[c]	Formula Intakes Per User (L/d)		Jennewein 2'-FL Intakes Per User (g/d)	
			Mean	90th Percentile	Mean	90th Percentile
Infants, 0-5 mo	89	99.3%	0.9	1.2	1.9	2.5
Infants, 6-11 mo	111	93.9%	0.8	1.2	1.7	2.4
Toddlers, 12-35 mo	13	7.2%	0.4	0.6	0.8	1.2

Notes:

[a] Breastfeeding infants and children were excluded from the sample population.

[b] Number of people consuming infant formula during the study period.

[c] Weighted percent.

Concentrations of 2'-FL in formula based on maximum target addition level of 2'-FL to formula at 2.0 g/L. Formula intake data are based on National Health and Nutrition Examination Surveys (NHANES) 2015-2016 survey. Because this is a daily average, some participants who had day 1 but not day 2 data are included using a single day of consumption.

Abbreviations: 2'-FL = 2'-fucosyllactose; d = day; g = grams; L = liters; mo = months.

PART 4. SELF-LIMITING LEVELS OF USE

The intended uses of Jennewein's 2'-FL are not self-limiting.

PART 5. EXPERIENCE BASED ON COMMON USE IN FOOD

5.1 Naturally-Occurring 2'-FL: History of Exposure and Use

Human breast milk is recommended as the first food for infants because it provides optimum nutrition and immunity benefits and reduces instances of disease later in life such as asthma, type 1 diabetes, and childhood leukemia *(*Agostoni *et al*., 2009; Field, 2005; Kunz *et al.,* 1999; van Rossum *et al.,* 2005). Breastfeeding is the preferred method for infant nutrition and is supported and promoted by professional pediatric organizations and federal health agencies. As previously described in GRN 000571 (Section 3, p. 19), most women naturally produce milk with 2'-FL and those that do not still produce fucosylated HMOs. 2'-FL occurs in many other mammals, particularly in the hominidae family, and appears to be highly conserved throughout human evolutionary development. Naturally occurring 2'-FL is one of the most abundant HMOs, present at mean quantities of approximately 1 – 3.5 g/L and making up approximately 20 – 30% of total HMOs (Coulet *et al*., 2014; Kunz *et al*., 2000). Therefore, most infants, including preterm infants and infants allergic to cow's milk, have a history of exposure to naturally produced 2'-FL.

5.1.1 Presence of 2'-FL in Human Milk

As 2'-FL is a substance found in the human milk of approximately 85% of the population worldwide, infants with food allergies and preterm infants are exposed to 2'-FL. Thus, hypoallergenic status and suitability for preterm infant consumption of products containing 2'-FL will be maintained with the addition of Jennewein 2'-FL.

2'-FL is present at mean quantities of approximately 1 – 3.5 g/L in human breast milk (Coulet *et al*. 2014; Kunz *et al*. 2000) and average levels range from 1.1 g/L to 3.4 g/L, depending on population (McGuire *et al*. 2017). Infants fed with breast milk from non-secretor mothers secrete 2'-FL in the urine and in the stool (Kunz and Rudloff 2017).

A targeted review focusing on publications investigating 2'-FL concentrations over the course of lactation and 2'-FL concentrations in the milk of women who gave birth to preterm or low birth weight babies was conducted. The findings of this review are presented in Part 5.1.2 and 5.1.3.

5.1.2 2'-FL Over the Course of Lactation

Thurl *et al*. (2010) found that 2'-FL concentration decreased from day 3 to day 90 of the lactation period from 4.1 to 2.6 g/L, respectively. Though the concentration of 2'-FL declines as lactation continues, the volume of breast milk consumed increases as the infant develops. Therefore, the amount of 2'-FL remains constant throughout the nursing period (Asakuma *et al*., 2008; Thurl *et al*., 2010).

In a systematic review conducted by Thurl *et al*. (2017), the authors compiled the concentration of 2'-FL over the course of lactation and found a decrease in the 2'-FL concentration by approximately 50% within the first 100 days after birth among term infants, and a similar decrease of 50% within the first 60 days after birth among pre-term infants. (Data for preterm infants were only available up to the first 60 days after birth.) This is based on seven studies of term infants (Asakuma *et al*. 2008, Coppa *et al*. 1999, Chaturvedi *et al*. 2001, Erney *et al*. 2000, Hong *et al*. 2014, Smilowitz *et al*. 2013, and Thurl *et al*. 2010 as cited in Thurl *et al*. 2017) and three studies of preterm infants (Gabrielli *et al*. 2011, Nakhla *et al*. 1999, and Van Niekerk *et al*. 2014 as cited in Thurl *et al*. 2017). The mean 2'-FL levels in mother's milk were similar for preterm and term infants between 0-30

days after birth. The mean 2'-FL level in mother's milk diverges at the 31-60 days after birth time period with the mean 2'-FL level among term infants higher than the mean level among preterm infants (Thurl *et al*. 2017). These studies demonstrate that 2'-FL is present over the course of lactation regardless of whether the infant was born preterm or at term.

5.1.3 2'-FL in the Milk Produced by Mothers with Infants Born Preterm or with Low Birth Weight

To investigate the safety of 2'-FL given to preterm or low birth weight infants, a targeted review was conducted. Published studies were reviewed that focused on 2'-FL in the breast milk of mothers who gave birth to preterm or low birth weight infants. This targeted review demonstrated the presence of 2'-FL in human milk given to premature infants and low birth weight infants.

For this targeted review, the search terms "2-FL", "2'-fucosyllactose", "2-fucosyllactose" combined with variations of "preterm" and "birth weight" were used in the United States National Library of Medicine at the National Institutes of Health PubMed database and Google Scholar. In Google Scholar, only the first few pages of search results were screened for relevance. Relevant studies were also identified from reviewing the references of potentially relevant publications.

Preterm

As reported in the systematic review conducted by Thurl *et al*. 2017, though 2'-FL levels vary among the individual mothers, the concentrations of 2'-FL in breast milk among mothers who delivered at term were similar to those among mothers who delivered preterm: 2.75 g/L during the first 100 days' lactation vs. 2.77 g/L during the first 60 days' lactation, respectively.

To illustrate the level of variability between milk-producing individuals and during lactation, the details of two of the preterm studies cited in Thurl *et al*. 2017 are described: Among 13 mothers who delivered at mean gestational age of 29.5 (standard deviation (SD) 3.1) weeks, the median concentration of 2'-FL in 23 samples collected within 60 days after birth was 1.1 g/L, compared with 1.3 g/L in three term milk samples (Nakhla *et al*. 1999). Among 35 positive secretor and Lewis genotypes) mothers of preterm infants (mean gestational age 27.9 weeks), the mean concentration of 2'-FL in milk decreased significantly ($p<0.05$) from day 4 postpartum to day 30: The mean 2'-FL concentration sampled at day 4 was 7.23 g/L 1.40 g/L at day 30 (Gabrielli *et al*. 2011). However, as mentioned in Part 6.1.2, the volume of intake of milk increases as the infant grows older, and the overall intake of 2'-FL per day is likely to be steady over the course of breastfeeding.

A recent study (Austin *et al*. 2019) analyzed 500 samples of milk from 28 mothers breastfeeding term infants and 25 mothers breastfeeding very preterm infants born at <32 weeks gestational age and weighing less than 1500 g at birth. Austin *et al*. concluded that the concentration of each HMO in the mother's milk was approximately the same for both preterm and term infants at the same postpartum age and lactation stage. However, stratified by Milk Group based on the levels of 2'-FL (a marker for FUT2 enzyme activity) and LNFP-II (a marker for FUT3 activity), the levels of HMOs in mother's milk were different for the mothers of preterm and term infants at the same postpartum age and lactation stage. In the Austin *et al*. study, "Milk Group 1" is defined as the group of mothers whose milk contains both active FUT2 and active FUT3 enzymes. Because of the presence of both FUT2 and FUT3, the milk contains the highest levels of 2'-FL. (This group is also the most

commonly found group in the population.) 2'-FL concentrations were significantly lower in the milk of mothers who gave birth to preterm infants when sampled at postpartum weeks 1-3 compared to the milk of mothers who gave birth at term. The difference was 0.7-0.8 g/L (20-27%) lower.

Austin *et al*. (2019) found that 2'-FL is present in the milk of mothers who gave birth to preterm infants (dependent on the presence of FUT2 and FUT3 enzymes) as with the milk of mothers who gave birth at term; however, at two equivalent developmental ages (postmenstrual age of weeks 39-43 and week 45), the 2'-FL concentrations were significantly lower in the milk of mothers who gave birth to preterm infants compared to milk of mothers who gave birth at term.

De Leoz *et al*. (2012) analyzed concentrations of 2'-FL in milk from 15 mothers who delivered preterm and 7 mothers who delivered term. The percentage of 2'-FL among HMOs did not differ significantly between mothers of term infants and mothers of preterm infants. However, there was greater fluctuation in 2'-FL concentrations within the group of mothers who delivered preterm. This study found variability of 2'-FL concentrations in mother's milk providing evidence that there is more variability in 2'-FL production among mothers who delivered preterm.

Thus, published studies support the finding that 2'-FL is commonly found in the milk of mothers who deliver preterm. Some studies show 2'-FL levels are the same in the milk of mothers who deliver preterm as in the milk of mothers who deliver at term; however, additional studies indicate that 2'-FL levels in milk of mothers who deliver preterm may be lower and more variable compared to the levels of 2'-FL in mothers who deliver full-term. For both mothers of preterm infants and mothers of term infants, the concentration of 2'-FL in milk declined over time (lactation postpartum days). Milk consumption is expected to increase with the growing infant, so the decline in 2'-FL concentration over time is expected to be countered by increased consumption of mother's milk and, therefore, the infant is ingesting a consistent EDI of 2'-FL.

Low birth weight

Few studies specifically captured the levels of 2'-FL in milk produced by mothers who gave birth to low birth weight, very low birth weight, or extremely low birth weight infants; however, infants born preterm (Part 6.1.2) are likely to fall into these low birth weight categories (i.e. Austin *et al*. 2019).

Wejryd *et al*. (2018) measured HMO levels in breast milk from 91 mothers to 106 extremely low birth weight infants during the neonatal period. Among this cohort, they reported a median of 5,390 µmol 2'-FL/L (2.63 g/L) milk at 14 days after delivery, 4,270 µmol/L at 28 days post-delivery (2.09 g/L), and slowly decreasing to 4,379 µmol/L (2.14 g/L) at the 36th post-menstrual week (Wejryd *et al*. 2018).

In a study (Van Niekerk *et al*. 2014) comparing HMO composition of milk from mothers with or without HIV and infants with or without necrotizing enterocolitis in South Africa, 2'-FL was measured in milk from the first month after birth among preterm, very low birthweight infants (≥500 and ≤1250 g at birth). Among 20 women with secretor status, the mean 2′-FL concentration in milk four days postpartum was 1.23 g/L and the mean 2'-FL concentration 28 days postpartum was 0.6 g/L (Van Niekerk *et al*. 2014). This study shows that 2'-FL is present in the milk of mothers who gave birth to preterm, very low birthweight infants.

Autran and colleagues (2017) measured 2'-FL and other HMOs in milk of 200 mothers who predominantly breastfed their extremely low birthweight infants in the first 28 days postpartum. Among eight cases (of necrotizing enterocolitis) and 40 matched controls (5 matched to each case), the mean concentration of 2'-FL in breastmilk fed to the infants was around 1 g/L (estimated from figure). There was no significant difference in 2'-FL concentrations between the two groups.

Thus, studies show that 2'-FL is present in the milk of most mothers who gave birth to low birth weight infants. Similar to the evidence focusing on 2'-FL in mother's milk of mothers who delivered preterm, authors of some studies concluded that 2'-FL levels were the same in milk produced by mothers who gave birth to low birth weight infants as in the milk produced by mothers who gave birth to normal-weight infants. Other studies, however, showed that 2'-FL levels were different in the milk of mothers who gave birth to low birth weight infants compared to mothers who gave birth to term infants.

5.2 Manufactured 2'-FL: Existing Exposure and Use

Food ingredient safety authorities in multiple jurisdictions have reviewed the safety of use of Jennewein 2'-FL as an ingredient added to infant formula, follow-on formula, and toddler formula and the ingredient currently is marketed in these regions (notably the United States (GRN 000571), the European Union (EFSA 2015), and Canada (Health Canada 2018)). Use levels range between 1.0 to 2.0 g 2'-FL per L infant formula as consumed. Since September 2016, Jennewein 2'-FL is found in multiple infant formula products worldwide. In the United States, addition of up to 2 g/L Jennewein 2'-FL, as consumed in non-exempt infant formulas and toddler formulas, is considered GRAS (GRN 000571). In addition to the United States, the European Union, and Canada, Jennewein 2'-FL currently is marketed in infant formulas and toddler formulas in approximately 25 countries worldwide including Mexico, Singapore, United Arab Emirates, and Israel.

PART 6. NARRATIVE

6.1 Introduction

Human breast milk is the preferred food for infants (US HHS, 2011). Human breast milk contains a variety of substances to support the rapid growth and development of infants in their first years of life including oligosaccharides. Human breast milk is obviously safe for infants; however, it is not always available. Therefore, quality formulas seek to replicate breast milk in nutritional content and chemical profile. Jennewein 2'-FL is a commercially produced version that is substantially chemically equivalent to the naturally occurring human milk oligosaccharide 2'-FL. In addition to the chemical equivalence of the Jennewein 2'-FL to naturally occurring 2'-FL, other factors must be addressed to demonstrate safety. Information regarding analytical testing to demonstrate the absence or acceptable levels of manufacturing residuals including chemical byproducts, processing aids, solvents, bacteria, bacterial proteins, recombinant DNA, and endotoxins is provided in GRN 000571 and Supplement 1. (as presented in GRN 000571, Section 2.3.2, p. 16 and Supplement 1, Appendix A).

As described in GRN 000571, 2'-FL is a substance found in the human milk of approximately 85% of the population worldwide. Thus, infants and toddlers with food allergies and infants born preterm are exposed to 2'-FL. Hypoallergenic status and suitability for preterm infant consumption of products containing 2'-FL will be maintained with the addition of Jennewein Biotechnologie 2'-FL. Furthermore, as described in Part 5.1.1, infants born to non-secretor mothers have 2'-FL in their urine and stool, evidence that infants are producing 2'-FL themselves.

In this part, the safety data from toxicological and clinical testing for Jennewein 2'-FL and other synthesized 2'-FL are presented and discussed.

6.2 Absorption, distribution, metabolism, and excretion of 2'-FL

As described in GRN 000571 (Section 6.2.1, p. 27-28), several studies have evaluated the absorption, distribution, metabolism and excretion (ADME) of human milk components, including human milk oligosaccharides. The focus on infant formula oligosaccharides has been as substrates for intestinal microflora, though studies have demonstrated that certain HMOs can be absorbed into the blood stream through the intestinal wall and excreted by the kidneys intact. At least 95% of ingested 2'-FL is directly available to gut microbiota and less than 5% is absorbed intact by infants. 2'-FL absorbed by infants enters the circulatory system and excreted in urine intact or minimally metabolized. Gut microbiota readily metabolize the unabsorbed 2'-FL into short-chain fatty acids.

In a clinical study testing chemically synthesized 2'-FL in infants with a human milk control (Marriage *et al.*, 2015), the mean plasma concentrations at day of life 42 were significantly different for each treatment group though the relative absorption of 2'-FL was comparable at 0.7% among infants fed formula containing 0.2 g 2'-FL/L, 0.05% among infants fed formula containing 1.0 g 2'-FL/L, and 0.05 among infants fed human milk. By day of life 119, the mean plasma concentrations between the two groups fed formula containing 2'-FL were not significantly different (data was not collected for the human milk-fed group at this time point). The plasma concentrations of 2'-FL decreased significantly for the 0.2 g 2'-FL/L formula group, the 1.0 g 2'-FL/L formula group, and the human milk groups ($p = 0.017$, 0.008 and 0.015, respectively) for day of life 42 to 119 and urine concentrations decreased significantly for the human milk-fed group ($p=0.018$) but did not change significantly for the

groups fed formula containing 2'-FL. Mean urine concentrations were significantly different among the groups, but relative excretion was similar among the groups fed human milk or formula containing 2'-FL: 1.35%, 1.50% (formula containing 0.2 g 2'-FL/L) and 1.26% (formula containing 1.0 g 2'-FL/L), respectively (Marriage *et al.*, 2015).

6.3 Safety of 2'-FL Demonstrated in Toxicological and Clinical Studies

Studies confirm that 2'-FL, biotechnologically produced and substantially chemically equivalent to 2'-FL isolated from human breast milk, is safe and suitable for its proposed uses. Based on evidence presented in genotoxicity studies (GRN 000571 Section 6.3.1 and 6.3.2, p. 28-29 and Supplement 1 Section 4.2.2.1 p. 9-10), oral toxicity studies in rats and piglets (GRN 000571 Section 6.3.3, p. 42-44 and Supplement 1 Section 4.2.2.2 p. 10-11), and clinical studies (Supplement 1 Section 4.2.2.3 p. 11-14), the Expert Panels concluded that Jennewein 2'-FL was safe, suitable and GRAS for the intended uses in non-exempt infant formula and toddler formula. The results from these studies of Jennewein 2'-FL and supporting studies of other biotechnologically produced 2'-FL are summarized briefly below and described in more detail in GRN 000571 (Section 6.3, p. 28-35) and Supplement 1 (Section 4.2.2 p. 9-14). A summary table of these studies is provided in **Appendix E** of this GRAS Notice.

6.3.1 Mutagenicity and Genotoxicity Studies

Numerous studies have shown evidence that 2'-FL is not genotoxic. Multiple bacterial reverse mutation tests in *Salmonella typhimurium* and *E. coli* and other gene mutation tests show no evidence of cytotoxicity nor mutagenicity of 2'-FL produced by chemical synthesis or microbial fermentation (GRN 000571 Section 6.3.1 and 6.3.2 p. 28-29, Supplement 1 – Section 4.2.2.1 p. 9-10). The concentrations of 2'-FL tested were up to 2000 or 5000 ug/mL with and without metabolic activation. No signs of cytotoxicity and no increases in revertant colony numbers were reported.

Jennewein 2'-FL was tested in a micronucleus test with cultured human peripheral lymphocytes with concentrations up to 5000 ug 2'-FL/mL medium for four hours. The test was conducted in the absence and presence of metabolic activation employing two exposure times (without S9) and one exposure time (with S9). There were no indications of chromosomal damage under the test conditions of the study (Appendix D). Multiple mammalian cell micronucleus tests with cultured human peripheral lymphocytes showed that 2'-FL produced by either chemical synthesis or microbial fermentation had no evidence of mutagenicity or cytotoxicity (Supplement 1 Section 4.2.2.1 p. 9-10).

A micronucleus test in rat bone marrow cells isolated from rats 24 to 48 hours administered a single dose of 500, 100, or 2000 mg/kg-bw 2'-FL by oral gavage showed that Jennewein 2'-FL is not mutagenic (GRN 000571 Section 6.3.2 p. 29)No signs of systemic toxicity were reported through the highest dose level of 2000 mg Jennewein 2'-FL per kg bw. Jennewein 2'-FL did not increase the incidence of micronucleated polychromatic erythrocytes at any of the three tested dose levels.

As described in GRN 000571 (Section 6.3.4.1 p. 32-33), a gene mutation test employed the thymidine kinase (TK) test to evaluate the potential of chemically synthesized 2'-FL (concentrations up to 5000 ug/mL) to induce gene mutations at the TK-locus of mouse lymphoma cells in both the absence and presence of S9 metabolic mix. 2'-FL did not induce any biologically relevant increases in mutant frequency in the absence or presence of S9-mix. No signs of cytotoxicity were noted at any of the concentrations tested.

6.3.2 Oral Toxicity Studies

6.3.2.1 2'-FL Does Not Induce Toxic Effects After Repeated Ingestion in Animals

As described in GRN 000571 (Section 6.3.3 p. 29-35) and Supplement 1 (Section 4.2.2.2 p. 10-11), repeated dose toxicity studies evaluating the safety of 2'-FL produced via microbial fermentation or chemical synthesis administered via oral gavage or in the diet showed that 2'-FL did not induce toxic effects after repeated ingestion (detailed summaries in Supplement 1 – Section 4.2.2.2 and GRN 571 – Section 6.3.3).

Two studies exposed test animals to 2'-FL produced via microbial fermentation processes by adding it at up to 10% in their diet for 90 days (GRN 000571 Section 6.3.3.2 p. 30-31 and Supplement 1 Section 4.2.2.2 p. 10: Van Berlo *et al.* 2018). The two studies reported that under the test conditions, 2'-FL did not induce toxic effects. No mortalities were observed, no significant or exposure-related changes were noted, except for some changes in organ weights (details in table in **Appendix X**). The no-observed-adverse-effect-levels (NOAEL) reported were the highest (or only) level tested: 7.25 g/kg-bw/day (Supplement 1 Section 4.2.2.2 p. 11) or 7.66 g/kg-bw/day (GRN 000571 Section 6.3.3.2 p. 31).

Two studies administered 2'-FL via oral gavage in rats ranging from 1000 to 6000 mg/kg-bw for 90 days (GRN 000571 Section 6.3.4.3 p. 34-35: Coulet *et al*. 2014 and Supplement 1 Section 4.2.2.2 p. 10-11: Penard *et al*. 2015 as cited in GRN 000650). Both studies determined a NOAEL OF 5 g/kg-bw/day. Another 90-day oral toxicity study administered an 8:1 ratio mixture of 2'-FL and difucsyllactose (DFL) via oral gavage to neonatal rats (Supplement 1 Section 4.2.2.2 p. 10-11: Phipps *et al.* 2018). Doses tested were 0, 1000, 3000, and 5000 mg/kg bw/day of 2'-FL/DFL. No treatment-related effects were observed in male or female rats and the authors concluded a NOAEL of 5 g/kg bw/day, the highest dose tested.

6.3.2.2 Jennewein 2'-FL Had No Toxic Effects in a Neonatal Pig Study

A pre-clinical, oral administration study of Jennewein 2'-FL investigated the effect of Jennewein 2'-FL on the health and development of neonatal piglets and observed no toxic effects after repeated ingestion of 2'-FL for 21 days (GRN 000571 Section 6.3.3.3 p. 31-32). Because the first three weeks of piglets' lives share many similarities with the first three months of development of human infants, including similarities in digestive enzymes, nutrient absorption, gut closure, gut transit time, dietary requirements, and microbial population, they are suitable models to study the impact of dietary compounds on the development of infants (Guilloteau *et al*., 2010). Daily dietary administration of 2'-FL at concentrations up to 2000 mg 2'-FL/L/day was well tolerated and did not produce any adverse treatment-related effects on growth and development. There were no reported adverse effects on body weight or food efficiency. No mortalities were reported. There were no Jennewein 2'-FL-related adverse effects reported on clinical pathology findings, gastrointestinal pH, and macroscopic and microscopic findings at terminal necropsy. The highest dose tested, 2000 mg 2'-FL/L/day, was well tolerated and corresponded to a consumption of 292 mg/kg/day in male piglets and 299 mg/kg/ day in females.

6.3.3 Clinical Studies

Several clinical studies demonstrate the safety and tolerance of synthesized 2'-FL in humans. These studies are summarized in Supplement 1 (Section 4.2.2.3 p. 11-14: Marriage *et al.* 2015, Elison *et al*. 2016; Kajzer *et al.* 2016 via Reverri *et al*. 2018; Puccio *et al.* 2017; Storm *et al*. 2019). 2'-FL is well tolerated in infants and there was no evidence for increased adverse events or alterations in normal growth. One clinical study (Supplement 1 p. 13-14: Elison *et al*. 2016) demonstrated that 2'-FL is safe and well tolerated at concentrations up to 20 g 2'-FL per day for 14 days in healthy adults.

6.3.3.1 2'-FL is Well Tolerated in Infants

Four studies evaluating the safety of 2'-FL when administered to infants in their diet observed no evidence for increased adverse events or alterations in normal growth (Supplement 1 Section 4.2.2.3, p. 11-14: Marriage *et al.* 2015; Puccio *et al.* 2017; Kajzer *et al.* 2016 via Reverri *et al*. 2018; Storm *et al*. 2019). The 2'-FL test concentrations ranged from 0.2 g 2'-FL/L formula up to 1.2 g 2'-FL/L formula. 2'-FL was combined with GOS (Marriage *et al.,* 2015), scFOS (Kajzer *et al*., 2016 via Reverri *et al*., 2018), LNnT (Puccio *et al.,* 2017) or in a 100% whey formula (Storm *et al.,* 2019).

2'-FL and GOS

As discussed in Supplement 1 (Section 4.2.2.3 p. 11-12: Marriage *et al*. 2015), chemically-synthesized 2'-FL is well-tolerated in human infants at concentrations up to 1.0 g 2'-FL/L (the highest exposure concentration tested in this study) and for up to four months of oral consumption with reported adverse effects at levels not significantly different from controls. Marriage *et al*. (2015) conducted a 119-day study to examine growth and tolerance by infants fed infant formulas of chemically-synthesized 2'-FL supplemented with a caloric density approximating human milk and to study the uptake of the 2'-FL. The formulas also contained galactooligosaccharides (GOS) to bring the prebiotic concentrations up to 2.4 g/L.

No significant differences between groups for weight, length or head circumference were observed. All tolerance metrics were comparable, and 2'-FL was present in the plasma and urine of infants fed 2'-FL though the concentration fed did not result in a significant difference in 2'-FL uptake between groups, including the human milk-fed infants.

No safety concerns were observed with any of the formulas containing 2'-FL. There were no significant differences in adverse events between the experimental groups and the control group based on percentages.

2'-FL and scFOS

As described in Supplement 1 (Section 4.2.2.3 p. 12-13: Kajzer *et al*. 2016 via Reverri *et al*., 2018), infant formula supplemented with up to 0.2 g/L 2'-FL and 0.2 g/L short-chain fructooligosaccharide (scFOS) was safe and well tolerated in infants. A prospective, randomized, multi-center, double blinded, controlled trial assessing gastrointestinal tolerance demonstrated that 0.2 g/L 2'-FL and 0.2 g/L scFOS was safe and well tolerated in healthy term infants given the test formula for 35 days compared to infants fed breast milk. There were no statistically significant differences in sex, ethnicity, race, gestational age, weight, length, or age at enrollment among the three groups, nor were there differences in anthropometric measures. There were no significant differences in mean rank stool consistency, formula intake, anthropometric measures, or percent feedings with spit-up or vomit associated with feedings across the test groups at 35 days of age.

2'-FL and LNnT

Infant formula containing synthesized 2'-FL and LNnT is safe, well-tolerated and supports age-appropriate growth. As reported in GRN 735 and described in Supplement 1 (Section 4.2.2.3 p. 13), Puccio *et al*. (2017) conducted a double-blind, randomized, controlled clinical trial in healthy, full-term infants to evaluate the effects of infant formula supplemented with two HMOs (2'-FL and LNnT) on infant growth, tolerance and morbidity. One hundred and seventy-five infants aged 0 to 14 days old were randomly assigned to either a treatment group receiving formula containing a combination of 2'-FL (1 – 1.2 g/L) and LNnT (0.5 – 0.6 g/L) (n = 88) or formula that did not contain either oligosaccharide (n = 86) for up to 6 months. Weight gain was similar for both the control and test groups. Digestive symptoms and behavioral patterns were also similar between the groups; exceptions included softer stools and fewer night-time wake-ups in the test group at two months. Infants fed formula containing 2'-FL and LNnT had significantly fewer parental reports of bronchitis through four and 12 months, antipyretics use through four months, lower respiratory tract infection through 12 months, and antibiotics use through six and 12 months.

2'-FL in 100% whey, partially hydrolyzed formula

100% whey, partially hydrolyzed infant formula with or without the addition of 0.25 g 2'-FL/L formula is safe and well tolerated based on the results of a study previously described in Supplement 1 (Section 4.2.2.3 p. 13). Storm *et al.* (2019) conducted a randomized, controlled multicenter study wherein healthy infants 14 days of age were randomized into two groups and fed formula made from partially hydrolyzed, 100% whey protein, with (test group) or without (control group) the addition of 0.25 2'-FL g/L for six weeks. Results indicated that Infant Gastrointestinal Symptom Questionnaire scores were similar at baseline and first visit at six weeks exposure. Stool frequency and consistency were similar among the test and control groups throughout the study.

6.3.3.2 Safety and Tolerance of 2'-FL in the Diets of Adults

As described in Supplement 1 (Section 4.2.2.3 p. 14), 2'-FL is safe and well tolerated at concentrations up to 20 g 2'-FL per day for 14 days in healthy adults. In a double-blind, parallel, randomized, placebo-controlled study, Elison *et al.* (2016) evaluated the effects of supplementing the diets of 100 healthy adults (19 – 57 years old) with 2'-FL produced via microbial fermentation and/or lacto-N-neotetraose (LNnT) for up to two weeks. Study participants (51 males and 49 females) received 5, 10 or 20 grams of either 2'-FL, LNnT or 2'-FL with LNnT (2:1 mass ratio) or 2 g of glucose as the placebo each day at breakfast.

Participants completed a self-administered form reporting on abdominal pain, indigestion, reflux, diarrhea, and constipation which are ranked from 1 (no discomfort) to 7 (very severe discomfort) (gastrointestinal symptom rating scale). Participants also recorded bowel movement frequency and stool consistency.

Forty-four participants reported a total of fifty-six mild adverse events. The adverse events were usually a combination of symptoms including flatulence, bloating, and constipation. The participants taking the highest doses of 2'-FL and LNnT reported the most adverse events, the most common of which were flatulence, stomach pain, diarrhea, loose stool and "rumbling". Participants receiving the highest dosages did not have statistically significant changes on the gastrointestinal symptom rating scale. Changes in the average number of daily bowel movements were small, though statistically higher in the 20 g 2'-FL, 20 g LNnT, and 5 g LNnT groups (increase of 0.3 movements per day compared to baseline) but the authors deemed this as clinically irrelevant. Participants receiving 20 g 2'-FL reported softer

stools as compared to baseline. It is difficult to discern whether the common gastrointestinal symptoms reported by study participants were due to treatment or due to normal variation and increased awareness of symptoms during the study period. All measured clinical chemistry and hematology parameters remained within normal ranges.

6.4 Clinical Study in Milk-Allergic Infants

Nowak-Wegrzyn *et al*. (2019) conducted a clinical study among infants and young children with cow's milk protein allergy assessing the hypoallergenicity and safety of an extensively hydrolyzed formula supplemented with 1.0 g/L 2'-FL and 0.5 g/L LNnT against a control formula without HMO. Infants, toddlers, and young children aged 2 months to 4 years were assessed by double-blind, placebo-controlled food challenges to both formulas and then, if both challenges were negative, were given at minimum 240 mL of the test formula per day for one week. There was one child with an allergic reaction in each of the test and control groups, whereas 63 out of 64 children in the modified intention-to-treat group tolerated the test formula for one week. This study provides evidence that a formula supplemented with 1.0 g/L 2'-FL and 0.5 g/L LNnT is hypoallergenic and suitable for use among infants, toddlers, and young children up to 4 years with cow's milk protein allergy. While the 2'-FL used in this study was produced by another manufacturer, this study demonstrates that, as expected, infants, toddlers, and young children with an allergy to cow's milk protein do not have any inherent sensitivity to 2'-FL.

6.5 Summary of Safety Information

6.5.1 Summary of Safety Data for Exempt IF

Jennewein has created 2'-FL that is substantially chemically equivalent to the naturally occurring HMO 2'-FL; this ingredient was determined to be GRAS in non-exempt, term infant formula at use level 2.0 g/L and received "no questions at this time" letters from FDA (Appendix B).

The resulting product consists of ≥90% 2'-FL, with residual amounts of common mono-, di-, tri-, and tetra-saccharides closely related to 2'-FL and which are also present in human milk (lactose, LDFT, 3-FL, fucose, glucose and galactose) or otherwise naturally present in the human body (fucosylgalactose). Jennewein 2'-FL is manufactured in accordance with current good manufacturing practice (cGMP) to meet strict specifications and the resulting product is well characterized through rigorous analytical testing.

Breastfeeding is the preferred method for infant nutrition and is supported and promoted by professional pediatric organizations and federal health agencies. Most women naturally produce milk with 2'-FL and those that do not do still produce fucosylated HMOs. 2'-FL is a major component of human breast milk but does not occur in bovine milk and there are no commercially available 2'-FL products. 2'-FL serves as a prebiotic for commensal gut bacteria which metabolize prebiotics into short-chain fatty acids used for energy by colonocytes, and to stimulate sodium and water absorption. Therefore, commercially available formulas used as breast milk replacements do not provide one of the most abundant components of breast milk. Most infants have a history of exposure to naturally produced 2'-FL through breast milk and no history of exposure to 2'-FL produced by other means. Jennewein 2'-FL is a commercially produced version that is substantially chemically equivalent to naturally occurring human milk 2'-FL.

Furthermore, Jennewein 2'-FL does not contain residual components from the manufacturing process with allergenic potential and there is no evidence to suggest that it may cause adverse effects in sensitive populations.

As a naturally occurring substance in human milk among approximately 85% of the population worldwide, infants with cow's milk allergy and preterm infants are exposed to 2'-FL. A review of the literature showed substantial evidence for naturally-occurring 2'-FL in the milk produced by mothers who delivered preterm and/or delivered babies of low birth weights. A clinical study testing hypoallergenicity of an extensively hydrolyzed infant formula containing manufactured 2'-FL among 63 infants, toddlers, and young children with cow's milk allergy concluded hypoallergenicity of the formula and suitability for use.

The absence of milk proteins in Jennewein 2'-FL was demonstrated in multiple laboratory tests. Hypoallergenic status and suitability for preterm infant consumption of products containing 2'-FL will be maintained with the addition of Jennewein 2'-FL.

6.5.2 Summary of Toxicological and Clinical Data

A safety assessment on the use of Jennewein 2'-FL in hypoallergenic and preterm exempt infant formula was conducted based on guidance from FDA including the necessary types of data regarding the ingredient, its intended use, and available safety data including data indicating the absence of protein, multiple rat and piglet feeding studies, and human clinical studies in infants and adults.

Human clinical studies in infants administered up to 1.2 g 2'-FL/L in formula report that the 2'-FL-containing formulas are well-tolerated. A clinical study with adults tested up to 20 g 2'-FL per day for 14 days. The group ingesting 20 g 2'-FL per day reported the greatest number of mild adverse events compared to the other dose groups. Participants receiving the highest dosages did not have statistically significant changes on the gastrointestinal symptom rating scale.

Furthermore, several studies demonstrated that 2'-FL produced via chemical synthesis or microbial fermentation is neither genotoxic nor mutagenic. Repeated dose oral toxicity studies with 2'-FL demonstrate that it is safe for consumption by rats at dietary fortification levels ranging from 5 to 7.7 g/kg·bw/day and was well tolerated by neonatal pigs at dietary fortification levels of 299 mg/kg·bw/d (2.0 g/L/day). In the 90-day oral toxicity study with Jennewein 2'-FL in rats, the NOAEL was determined to be 7.66 g/kg·bw/day (GRN 000571 Section 6.3.3 p. 31).

To extrapolate this level of 2'-FL to infant and toddler consumption, a 100-fold safety factor (10 for interspecies variability and 10 for intraspecies variability) is applied to the 7.66 g/kg-bw/day NOAEL from the 90-day oral toxicity study. This results in a consumption level of 76.6 mg 2'-FL/kg-bw/day. Assuming an average body weight of 6.8 kg, an infant 0 to 6 months old would consume 520 mg 2'-FL/day. Assuming an average weight of 9.3 kg, an infant 7 to 12 months old would consume 710 mg 2'-FL/day. Toddlers 1 to 3 years old would consume 1000 mg 2'-FL/day based on an average body weight of 13.8 kg.

6.5.3 Jennewein 2'-FL Intake Compared with Background 2'-FL Intake

The occurrence of 2'-FL in human milk is variable and ranges from 1.1 to 3.4 g 2'-FL per L human milk (as discussed in Part 5).

As described in Part 6.5.2, the level of intake based on the NOAEL determined in the 90-day oral toxicity study in rats is lower than the level of intake of 2'-FL as consumed in human milk, assuming consumption of approximately 1 L of milk per day for newborns (based on NHANES 2013-2016 estimated daily intake of formula).

As described in Part 3.5, assuming an addition level of 2 g Jennewein 2'-FL per L of formula, the mean intake level for infants 0 to 5 months old is 1.9 g/day. The mean intake level for infants 6 to 11 months old is 1.7 g/day. The mean intake level for toddlers is 0.8 g/day. The 2 g Jennewein 2'-FL per L of formula is within the naturally occurring range of 2'-FL in human milk (1.1 to 3.4 g 2'-FL per L) and accordingly, the intake level is also within the range of background 2'-FL intake levels. Jennewein and the Expert Panel determined that the 2 g 2'-FL/L level in infant formula and toddler formula is safe and suitable.

6.6 Expert Panel Conclusions

Jennewein determined that Jennewein 2'-FL is GRAS for use in exempt infant formula (i.e. preterm and hypoallergenic uses) and hypoallergenic toddler formula on the basis of scientific procedures. A panel of experts (Expert Panel) evaluated the safety data described herein and determined that the intended uses of Jennewein 2'-FL are safe, suitable and GRAS. The Expert Panel, independently and collectively, critically evaluated the manufacturing and characterization data for Jennewein 2'-FL, the intended use information including levels of intake, the available safety data presented herein. The Expert Panel determined that the suitability and safety of the proposed uses of Jennewein 2'-FL are supported by the appropriate, publicly available, scientific data. Based on scientific procedures, the Expert Panel unanimously concluded that the proposed uses of 2'-FL in infant formula for preterm use and hypoallergenic use and for toddler formula for hypoallergenic use, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting the food grade specifications presented herein, are safe and suitable. Further, the Expert Panel concluded that Jennewein 2'-FL manufactured consistent with cGMP and meeting the food-grade specifications presented herein are GRAS for the proposed uses based on scientific procedures and that other experts, qualified by scientific training and experience, and evaluating the same data and information would concur with their conclusions. A summary of the Expert Panel's conclusion and the data and information upon which their conclusion was based is presented in Appendix A.

6.7 Conclusion of GRAS Status

The suitability and safety of the proposed uses of Jennewein 2'-FL are supported by the appropriate, publicly available, scientific data. The proposed uses of Jennewein 2'-FL in hypoallergenic infant formulas and toddler formulas and in preterm infant formulas manufactured with cGMP and meeting the specifications presented herein are safe and suitable. These uses are GRAS based on scientific procedures and it is our opinion that other experts qualified by scientific training and experience and evaluating the same data and information would concur with these conclusions.

PART 7. LIST OF SUPPORTING DATA AND INFORMATION

7.1 Acronyms and Abbreviations

21 CFR	Part 21 of the Code of Federal Regulations
2'-FL	2'-fucosyllactose
3-FL	3-fucosyllactose
ADME	absorption, distribution, metabolism, excretion
AMU	atomic mass unit
bw	body weight
CASRN	Chemical Abstracts Service Registry Number
CFSAN	Center for Food Safety and Nutrition
cfu	colony-forming units
cGMP	current good manufacturing practice
cm	centimeter
d	day(s)
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDI	estimated daily intake
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
ESI	electro spray ionization
EU	endotoxin unit
FDA	United States Food and Drug Administration
FFDCA	Federal Food, Drug and Cosmetic Act
FOIA	Freedom of Information Act
FUT2	fucosyltransferase 2
FUT3	fucosyltransferase 3
g	gram(s)
GDP-fucose	guanosine 5'-diphospho-fucose
GOS	galactooligosaccharide
GRAS	Generally Recognized as Safe
GRN	Generally Recognized as Safe notice
HMO	human milk oligosaccharide
HPAEC/PAD	high-performance anion-exchange chromatography with pulsed amperometric detection
ISO	International Organization for Standardization
kDa	kilodalton
kg	kilogram(s)
L	liter(s)
LC-MS/MS	liquid chromatography – mass spectrometry
LDFT	lactodifucotetraose
LNFP	lacto-*N*-fucopentaose

LNnT	lacto-*N*-neotetraose
M	Molar
mg	millgram(s)
mL	milliliter(s)
mm	millimeter(s)
mol	mole
mS	millisiemens
MW	molecular weight
ng	nanogram(s)
NHANES	National Health and Nutrition Examination Survey
NOAEL	no observed adverse effect level
OFAS	Office for Food Additive Safety
qPCR	quantitative real-time polymerase chain reaction
µg	microgram(s)
µmol	micromole(s)
scFOS	short-chain fructooligosaccharides
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TK	thymidine kinase
ug	microgram(s)
UV	ultraviolet

7.2 References

All references listed here are generally available. As this GRN references GRN 000571 and Supplement 1 to GRN 000571, references not previously included (i.e. new to this GRN) are indicated with an asterisk (*). These new references are provided as PDFs in Appendix F.

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7.3 Appendices

Appendix A: Expert Panel Consensus Statement

Appendix B: Agency Response Letters

Appendix C: Protein Analyses

Appendix D: Jennewein 2'-FL Micronucleus Test (Cultured Human Peripheral Lymphocytes)

Appendix E: Previously Reviewed Safety Studies in GRN 000571 and Supplement 1 to GRN 000571

Appendix F: References

APPENDIX A
EXPERT PANEL CONSENSUS STATEMENT

Expert Panel Consensus Statement on the Generally Recognized as Safe Status of Proposed Uses of 2′-Fucosyllactose

Ramboll Environment and Health (Ramboll), on behalf of Jennewein Biotechnologie, GmbH (Jennewein) convened a panel of experts (Expert Panel), qualified by their scientific training and experience to evaluate the safety of food ingredients, to determine the safety, suitability and the Generally Recognized as Safe (GRAS) status of exempt infant formula use of 2′-fucosyllactose (2′-FL), a human milk oligosaccharide (HMO), manufactured using a genetically engineered *Escherichia coli* (*E. coli*) BL21 (DE3) strain (a strain of the commensal bacterium *E. coli* BL21) as a processing aid. The Expert Panel included Joseph V. Rodricks, PhD, DABT; Judith K. Jones, MD, PhD; and Gavin P. Thompson, PhD.

The Expert Panel, independently and collectively, critically evaluated the available information presented in documents prepared and presented by Ramboll and other materials deemed appropriate and necessary for this review. This information included the description of the substance (including the identity and physical and chemical properties), analyses demonstrating and confirming the purity and manufacturing consistency of the product, the chemical identity of 2′-FL, and product specifications. Information on safe use of production strains and analyses demonstrating the absence of recombinant DNA or proteins were provided and reviewed. A critical overview about the history of use, intended conditions of use and levels of use, its regulatory status, and anticipated exposures or intake, product stability and safety assessment of the Jennewein 2′-FL ingredient were provided to and reviewed by the Expert Panel.

Following its independent and collective critical evaluation of the available information, the Expert Panel, convened on 27 August 2019 with representatives of Jennewein. Following the discussion, the Expert Panel unanimously agreed to the conclusions described herein. A summary of the basis for these conclusions follows.

Description of 2′-FL, the Manufacturing Process, and Product Specifications

The substance in this GRAS determination is 2′-fucosyllactose (2′-FL). 2′-FL is a naturally occurring component of human breast milk and one the most abundant human milk oligosaccharides (HMOs). The Chemical Abstracts Service Registry Number for 2′-FL is 41263-94-9.

A GRAS notification (GRN) 571 for Jennewein 2′-FL for use in non-exempt, milk-based term infant formulas and in toddler formulas at a maximum level of 2 g/L as consumed was submitted to FDA and received a "no questions at this time" Agency Response Letter in 2015. Jennewein 2′-FL been on the market in the United States in nonexempt infant formulas and toddler formulas since 2016. The commercially produced biosynthesized, purified 2′-FL is manufactured by Jennewein via a fermentation process using a genetically engineered non-pathogenic bacterial strain, *E. coli* BL21 (DE3), as a processing aid. *E. coli* BL21 (DE3) has been used previously to manufacture an enzyme that has received a "no questions at this time" letter from FDA (GRN 485).

As described in GRN 571, Jennewein 2′-FL product with identical sugar compositions will be commercially available as a spray-dried lyophilized powder and a liquid concentrate with a 45% 2′-FL content. The Jennewein 2′-FL ingredient consists of a minimum of 90% 2'-FL, and concentrations of less than or equal to 5% each of common mono-, di-, and trisaccharides closely related to 2′-FL and also present in human milk (lactose, LDFT, 3-FL, fucose, glucose and galactose) or otherwise naturally present in the human body (fucosylgalactose). Jennewein used established NMR and LC-MS/MS analytical techniques to confirm that the identity and structure of Jennewein 2'-FL is the same as human breast milk 2′-FL. These data demonstrate that the chemical and physical properties of Jennewein 2′-FL are substantially chemically equivalent to human breast milk 2′-FL.

To ensure that a consistent food-grade ingredient is produced, Jennewein has established specifications for their 2'-FL ingredient. The chemical, physical and microbiological specifications of both the Jennewein 2′-FL powder and concentrate are presented in Table 1. Five batches were analyzed with regard to the chemical and microbiological parameters listed in the specifications including heavy metals (arsenic ≤ 0.2 mg/kg, cadmium ≤ 0.1 mg/kg, lead ≤ 0.02 mg/kg, and mercury ≤ 0.5 mg/kg), endotoxins (≤ 300 EU/g), aflatoxin (≤ 0.025 ug/kg), and microbial contamination (yeasts and molds meet specifications, and coliform, *Salmonella*, and *Cronobacter sakazakii* all absent). A quantitative real-time polymerase chain reaction (qPCR) confirmation test method was developed and validated by GeneCon International, GmbH in co-operation with Jennewein to detect the antibiotic genes used in the metabolic engineering of the *E. coli* BL21 (DE3) #1540 production strain. This confirmed that the Jennewein 2'-FL ingredient was devoid of recombinant genetic material. All tested batches for both the powdered and concentrate met the established specifications demonstrating that the Jennewein 2'-FL ingredient complies with appropriate specifications for food-grade materials and that a consistent product can be and is produced. The powdered ingredient is tested for aflatoxins, endotoxins, and GMO material. The concentrate ingredient is made from the powdered ingredient.

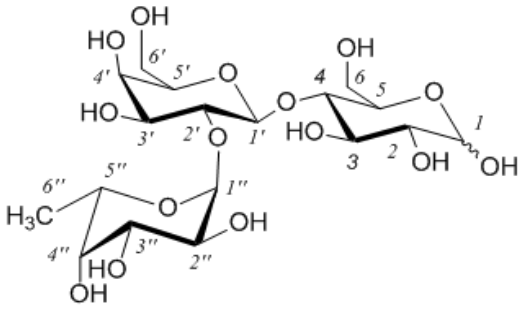

Figure 1. Chemical Structure of 2'-FL
Source: Jennewein Biotechnologie, GmbH

Stability tests confirmed that the Jennewein 2'-FL powder has a guaranteed shelf-life of at least two years (104 weeks) when stored under standard conditions of 25 °C and 65% humidity, and a shelf-life no less than six months (26 weeks) at 40 °C and 75% humidity in its original packaging. A shelf-life of no less than six months is also guaranteed for the Jennewein 2'-FL concentrate stored under standard conditions.

Absence of all potential allergenic proteins was demonstrated in batches of Jennewein 2′-FL three different ways. A silver stain sodium dodecyl sulfate-polyacrylamide gel electrophoresis test with the ability to detect 0.2 to 0.6 ng of protein per band confirmed the lack of protein in the 2′-FL batches. No detectable immunologically active casein and immunologically active whey protein was detected using enzyme-linked immunosorbent assays. Lastly, 2′-FL was tested for intact protein by size exclusion chromatography and no intact protein was detected.

History of Exposure and Use

Humans are exposed to fucosylated oligosaccharides such as 2′-FL and 3-FL while nursing as infants. Most women naturally produce milk with 2′-FL and those that do not produce other fucosylated HMOs. 2′-FL serves as a prebiotic for commensal gut bacteria which metabolize prebiotics into short-chain fatty acids used for energy by colonocytes, and to stimulate sodium and water absorption. 2′-FL occurs in many other mammals, particularly in the Hominidae family, and appears to be highly conserved throughout human evolutionary development. There are no commercially available 2′-FL products. Therefore, most people have a history of exposure to naturally produced 2′-FL at infancy.

2′-FL is a substance found in the human milk of approximately 85% of the population worldwide, including mothers who gave birth preterm. Preterm infants and infants with cow's milk protein allergy are exposed to 2′-FL in human milk. Thus, hypoallergenic status and suitability for preterm infant consumption of products containing 2′-FL will be maintained with the addition of Jennewein Biotechnologie 2′-FL. Jennewein 2′-FL is a commercially produced version that is substantially chemically equivalent to naturally occurring human milk 2′-FL.

Table 1. Specifications of Jennewein 2'-Fucosyllactose Powder and Liquid Concentrate

Parameter	Powder Product Specification	Concentrate Product Specification	Method
Chemical			
Solids content	N/A	45% w/v (± 5% w/v) dry matter in water	Dry weight after freeze-drying
Water content	≤ 9.0%	N/A	Karl-Fischer titration
Protein content	≤ 100 µg/g	≤ 100 µg/g freeze-dried matter	Nanoquant (modified Bradford)
Total Ash	≤ 0.5%	≤ 0.5% freeze-dried matter	ASU L 06.00-4 (a)
Arsenic	≤ 0.2 mg/kg	≤ 0.2 mg/kg freeze-dried matter	ASU L 12.00-06 (a)
Cadmium	≤ 0.1 mg/kg	≤ 0.1 mg/kg freeze-dried matter	ASU L 00.00-19/3 (a)
Lead	≤ 0.02 mg/kg	≤ 0.02 mg/kg freeze-dried matter	ASU L 00.00-19/3 (a)
Mercury	≤ 0.5 mg/kg	≤ 0.5 mg/kg freeze-dried matter	ASU 00.00-19/4 (a)
Aflatoxin M_1	≤ 0.025 µg/kg	N/A	DIN EN ISO 14501
Endotoxins	≤ 300 EU/g	N/A	Ph. Eur. 2.6.14
GMO detection	negative	N/A	qPCR
Carbohydrate content			
2'-Fucosyllactose	≥ 90% (Area)	≥ 90% (Area)	HPAEC-PAD
Lactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
3-Fucosyllactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
Difucosyllactose	≤ 5% (Area)	≤ 5% (Area)	HPAEC-PAD
Fucosyl-Galactose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Glucose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Galactose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Fucose	≤ 3% (Area)	≤ 3% (Area)	HPAEC-PAD
Microbiology analysis			
Standard Plate Count	≤ 10000 cfu/g	≤ 5000 cfu/g	ISO 4833-2
Yeast and Mold	≤ 100 cfu/g	≤ 50 cfu/g	ISO 21527-2
Coliform / Enterobacteriaceae	absent in 11 g	absent in 22 ml	ISO 4832 / ISO 21528-2
Salmonella	absent in 100 g	absent in 200 ml	ISO 6579
Cronobacter sakazakii	absent in 100 g	absent in 200 ml	ISO/TS 22964

Source: Jennewein Biotechnologie, GmbH
Abbreviations: ASU = Official collection of determination methods according to § 64 LFGB; cfu = colony-forming units; DIN EN ISO 14501 = German Institute for Standardization Milk and milk powder - Determination of aflatoxin M1 content - Clean-up by immunoaffinity chromatography and determination by high-performance liquid chromatography; EU = endotoxin units; HPAEC-PAD = High-performance anion-exchange chromatography with pulsed amperometric detection; ISO = International Organization for Standardization; ISO/TS = International Organization for Standardization Technical Specifications; LFGB = German Code on food and feed; N/A = Not Available; Ph. Eur = European Pharmacopoeia; qPCR = quantitative polymerase chain reaction.

Intended Use: Proposed Uses and Estimated Daily Intakes

Jennewein intends to use Jennewein 2'-FL as a prebiotic ingredient in exempt infant formulas for preterm infants and exempt infant formulas for hypoallergenic use beginning at birth in addition to nonexempt infant formulas and toddler formulas described in GRN 571. The maximum target use of the ingredient in exempt and nonexempt infant formulas and toddler formulas is two grams (g) of 2'-FL per liter (L) of formula as consumed. Published scientific literature demonstrates that 2'-FL used at a level of 2 g/L corresponds to the mean concentration of 2'-FL normally found in breast milk.

Based on the proposed maximum use of 2 g Jennewein 2'-FL/L in exempt and nonexempt infant formulas for term infants and toddler formulas, 0-5 months old infants were found to have the highest (1.8 g/d) estimated mean intake of Jennewein 2'-FL, with an estimated intake of 2.5 g/day for the 90th percentile (Table 2). A small number of infants consume formulas after the first year of life, and formula intakes are lower than intakes by infants 0-5 or 6-11 months of age. The estimated mean and 90th percentile 2-day average intakes of 2'-FL by toddlers, the target consumers of toddler formulas, are 1.1 and 2.0 g/day, respectively (Table 2).

The estimates of potential ingredient intake by infants were based on the survey population of non-breastfeeding infants. The youngest infants (i.e., prior to introduction of weaning foods) in the sample population therefore are presumably consuming only infant formula. Some infants, however, consume a combination of human milk and infant formula. Because the use level of 2 g/L corresponds to the mean concentration of 2'-FL normally found in breast milk, the overall intake of 2'-FL by combination breast milk/formula fed infants would likely be comparable to the intake of 2'-FL by infants receiving breast milk only.

Table 2. Estimated Daily Intake of 2'-FL from Infant and Toddler Formulas

			Formula Intake Per User (L/d)		Jennewein 2'-FL Intakes Per User (g/d)	
Population[a]	n[b]	Percent users[c]	Mean	90th Percentile	Mean	90th Percentile
Infants, 0-5 mo	141	100	0.9	1.2	1.8	2.5
Infants, 6-11 mo	142	86.3	0.8	1.1	1.6	2.2
Toddlers, 12-35 mo	20	3.8	0.5	1.0	1.1	2.0

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

Intended Effect

In accordance with 21 CFR 170.3(o)(20), the intended effect of adding the ingredient to food is as a nutrient necessary for the body's nutritional and metabolic processes. Jennewein 2'-FL is a prebiotic for commensal gut bacteria. These bacteria metabolize prebiotics into short-chain fatty acids, which are used by colonocytes in energy production and as a stimulant for sodium and water absorption.

Safety Assessment

In addition to GRN 571, numerous GRNs for non-HMO oligosaccharides have been submitted to FDA. These publicly available oligosaccharide GRNs, including GRNs: 489 and 392 (galacto-oligosaccharide or GOS), GRN 44 (fructo-oligosaccharide or FOS), and GRN 477 (long chain-inulin), have provided

comprehensive reviews about the effects of oligosaccharides on intestinal microflora. These publicly-available documents received no objection from FDA and provide an overview of the role of oligosaccharides as prebiotics for intestinal microflora.

Absorption, Distribution, Metabolism and Excretion

Several studies have evaluated the absorption, distribution, metabolism and excretion (ADME) of human milk components, including human milk oligosaccharides, though no studies were identified that evaluated the ADME of isolated, non-maternal 2′-FL. The focus on oligosaccharides has been as substrates for intestinal microflora, though studies have demonstrated that certain HMOs can be absorbed into the blood stream through the intestinal wall and excreted intact by the kidneys. Over 95% of ingested 2′-FL is directly available to gut microbiota and less than 5% is absorbed intact by infants. Gut microbiota readily metabolize the 2′-FL into short-chain fatty acids.

Toxicological Studies

The toxicological studies performed with Jennewein 2′-FL demonstrate the safety of the substance. Studies with Jennewein 2′-FL demonstrate that it is safe for consumption by rats at dietary fortification levels of up to 7.7 g/kg/day and was well tolerated by neonatal pigs at dietary fortification levels of 299 mg/kg/d (2.0 g/L/day) and no differences in the development of the piglets between control and test groups or any substance-related adverse events were reported.

Jennewein 2′-FL is neither mutagenic nor genotoxic. Jennewein 2′-FL does not contain residual components or impurities from the manufacturing process with allergenic potential and there is no evidence to suggest that it may cause adverse effects in sensitive populations. Residual components in the Jennewein 2′-FL ingredient are also present in human milk (lactose, LDFT, 3-FL, fucose, glucose and galactose) or otherwise naturally present in the human body (fucosylgalactose) and were present in the material used in the Jennewein 2′-FL toxicological studies.

Clinical Study in Milk-Allergic Infants

A clinical study testing hypoallergenicity of an extensively hydrolyzed infant formula containing manufactured 2′-FL among 63 infants, toddlers, and young children with cow's milk allergy concluded hypoallergenicity of the formula and suitability for use. The control formula was an extensively hydrolyzed infant formula without HMO supplementation.

A critical evaluation of the available evidence indicates that infant formulas containing up to 2.0 g Jennewein 2′-FL/L are safe and suitable for term infants from birth and that toddler formulas containing up to 2.0 g Jennewein 2′-FL/L are safe and suitable for children 12 to 35 months of age.

Conclusions

We, the members of the Expert Panel, have independently and collectively, critically evaluated the available information on 2′-fucosyllactose (2′-FL) manufactured by Jennewein Biotechnologie, GmbH using a genetically engineered *E. coli* BL21 (DE3) strain as a processing aid (presented in the dossier prepared by Ramboll on behalf of Jennewein and summarized above) and other information deemed appropriate and we unanimously conclude that the proposed uses of Jennewein 2′-FL, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting the food grade specifications presented in the dossier, are safe and suitable.

We further unanimously conclude that the proposed use of Jennewein 2′-FL in exempt infant formulas, manufactured consistent with current Good Manufacturing Practice (cGMP) and meeting the food grade specifications presented in the dossier, are Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our opinion that other experts, qualified by scientific training and experience, and evaluating the same data and information, would concur with these conclusions.

Joseph V. Rodricks, PhD, DABT
Principal
Ramboll Environment & Health
Arlington, Virginia

Signature: [redacted]

Date: 6 Sept 12, 2019

Judith K. Jones, MD, PhD
Principal Consultant
PER-Pharmacoepidemiology
Pharmacovigilance & Risk Management
Pharmalex US Inc.
Fairfax, Virginia

Signature: [redacted]

Date: Sept 20, 2019

Product Chemistry Advisor:

Gavin P. Thompson, PhD
Principal Consultant
Ramboll Environment & Health
Phoenix, Arizona

Signature: [redacted]

Date: 16 Sep 2019

APPENDIX B
AGENCY RESPONSE LETTERS: GRN 571

Agency Response Letter GRAS Notice No. GRN 000571

Return to inventory listing: **GRAS Notice Inventory (https://wayback.archive-it.org/7993/20171031001515/http://www.fda.gov/grasnoticeinventory)**

See also **Generally Recognized as Safe (GRAS) (https://wayback.archive-it.org/7993/20171031001515/http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/default.htm)** and **about the GRAS Notice Inventory (https://wayback.archive-it.org/7993/20171031001515/http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)**

CFSAN/Office of Food Additive Safety

November 6, 2015

Gavin Thompson, Ph.D.
Ramboll Environ
2111 East Highland Ave., Suite 402
Phoenix, AZ 85016

Re: GRAS Notice No. GRN 000571

Dear Dr. Thompson:

The Food and Drug Administration (FDA) is responding to the notice, dated March 2, 2015, that you submitted on behalf of Jennewein Biotechnologie, GmbH (Jennewein) in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS); the GRAS proposal). FDA received the notice on March 4, 2015, filed it on March 20, 2015, and designated it as GRAS Notice No. GRN 000571.

The subject of the notice is 2'-fucosyllactose (2'-FL). The notice informs FDA of the view of Jennewein that 2′-FL is GRAS, through scientific procedures, for use as an ingredient in non-exempt, milk-based term infant formulas and in toddler formulas at a maximum level of 2 grams per liter (g/L) of reconstituted formula.

As part of its notice, Jennewein includes the report of a panel of individuals (Jennewein's GRAS panel) that evaluated the data and information that are the basis for Jennewein's GRAS determination. Jennewein considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Jennewein's GRAS panel evaluated the identity, manufacturing process, specifications, estimated dietary exposure, and published information supporting the safety of 2'-FL. Based on this review, Jennewein's GRAS panel concluded that 2'-FL, produced in accordance with good manufacturing practices, is GRAS under the conditions of its intended use.

Jennewein discusses the identity of 2'-FL. The chemical name is α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranoside and the CAS Registry Number is 41263-94-9. Jennewein describes two formulations of 2'-FL, a white to ivory colored powder (≥90% 2'-FL) and a liquid concentrate (≥45% 2'-FL). Jennewein concludes that 2'-FL manufactured by the process described in the notice is chemically and structurally equivalent to that present in human milk.

Jennewein describes the method of manufacture for 2'-FL and provides information on the nonpathogenic and nontoxigenic production organism, *Escherichia coli* BL21 (DE3) #1540.[1] First, the production organism is inoculated into a fermentation medium that contains lactose. The fermentation process is continued until a specified level of 2′-FL is produced. The culture supernatant containing 2′-FL is separated from the microbial biomass by filtration. The filtrate is subjected to a series of cationic and anionic ion exchange resins to remove impurities (e.g., proteins, DNA, organic acids, and inorganic salts). The eluent is concentrated by evaporation and decolorized with activated carbon. The eluent is then subjected to electrodialysis and additional ion exchange resins, and treatment with activated carbon. The 2′-FL solution is then subjected to simulated moving bed (SMB) chromatography with cationic ion exchange resin and aqueous ethanol. The pH of the resulting solution is adjusted with sodium hydroxide and is then subjected to an ion exchange resin, followed by evaporation, electrodialysis, concentration, and filtration. This final solution is packaged as 2′-FL concentrate or spray-dried to produce a powder. Jennewein states that all processing aids used in the manufacture of 2'-FL are food-grade.

Jennewein provides specifications for the 2'-FL spray-dried powder and the 2'-FL liquid concentrate. Specifications for the spray-dried powder include minimum levels of 2′-FL (≥ 90%) and limits on moisture (≤ 9 %). Specifications for the liquid concentrate include the level of dry matter (45% w/v ±5%). Additional specifications for both formulations include limits for lead (≤ 0.02 milligrams per kilogram (mg/kg)), arsenic (≤ 0.2 mg/kg), cadmium (≤ 0.1 mg/kg), mercury (≤ 0.5 mg/kg), proteins (≤ 100 mg/kg), and limits on microbial contaminants including no detectable *Cronobacter sakazakii* in a 100 gram (g) sample of spray-dried powder or 200 milliliter sample of liquid concentrate. Specifications for both formulations also include limits for other saccharides (on a percent of the carbohydrate fraction basis) including lactose (≤ 5%), 3-fucosyllactose (≤ 5%), difucosyllactose (≤ 5%), fucosylgalactose (≤ 3%), glucose (≤ 3%), galactose (≤ 3%), and fucose (≤ 3%). Jennewein provides the results of batch analyses of five lots of the spray-dried and liquid concentrate formulations of 2′-FL to demonstrate compliance with specifications.

Jennewein estimates the dietary exposure to 2′-FL based on the maximum intended use level and consumption data from the 2009-2010 National Health and Nutrition Examination Survey (NHANES). Jennewein states that the highest mean and 90th percentile dietary exposures to 2′-FL are in infants aged 0-5 months (1.8 and 2.5g/person/day (d), respectively). The mean and 90th percentile dietary exposures to infants aged 6 to 11 months are reported to be 1.6 and 2.2 g/person/d, respectively. Jennewein states that the mean and 90th percentile exposures to toddlers (12 to 35 months of age) are 1.1 and 2.0 g/person/d, respectively.

Jennewein discusses data and information supporting the safety of 2'-FL. Jennewein states that published studies in breast-fed human infants demonstrate that 2'-FL does not undergo significant digestion in the upper gastrointestinal tract. 2'-FL undergoes fermentation, and only a small fraction is absorbed intact and eventually excreted in urine. Jennewein discusses published 14-day dose-range finding and 90-day toxicity studies conducted in juvenile Wistar [Crl:WI(Han)] rats, and two *in vitro* mutagenicity studies. These studies were conducted using 2'-FL from a different source. In the 90-day study, administration by gavage of up to 5000 mg 2'-FL/kg body weight

(bw)/d to the test groups, and 6000 mg fructo-oligosaccharide/kg bw/d to the reference control group was well tolerated and did not elicit any treatment-related, toxicologically relevant effects. Additionally, Jennewein discusses a published 3-week dietary toxicity study conducted in neonatal piglets using its 2'-FL. Jennewein concludes that up to 292 mg 2'-FL/kg bw/d in males, and 299 mg 2'-FL/kg bw/d in females, did not result in any adverse health effects and did not impact piglet growth. Jennewein also discusses an unpublished 90-day dietary toxicity study in rats, an *in vitro* mutagenicity study, and a genotoxicity study conducted using its 2'-FL. The results of the unpublished 90-day toxicity study corroborate the safety conclusions of the published studies. Jennewein also concludes that 2'-FL is non-mutagenic as demonstrated by both the published and unpublished *in vitro* mutagenicity studies. Based on the totality of the data and information discussed in the notice, Jennewein concludes that 2'-FL is GRAS under the intended conditions of use.

Potential Labeling Issues

In describing the intended uses of 2'-FL and in describing the information that Jennewein relies on to conclude that 2'-FL is GRAS under the conditions of its intended use, Jennewein raises a potential issue under the labeling provisions of the Federal Food, Drug, and Cosmetic Act (FD&C Act). Under section 403(a) of the FD&C Act, a food is misbranded if its labeling is false or misleading in any particular. Section 403(r) of the FD&C Act lays out the statutory framework for the use of labeling claims that characterize the level of a nutrient in a food or that characterize the relationship of a nutrient to a disease or health-related condition. If infant formula products that contain 2'-FL bear any claims on the label or in labeling, such claims are the purview of the Office of Nutrition, Labeling, and Dietary Supplements (ONLDS) in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety neither consulted with ONLDS on this labeling issue nor evaluated the information in your notice to determine whether it would support any claims made about 2'-FL on the label or in labeling.

Intended Use in Infant Formula

Under section 412 of the FD&C Act, a manufacturer of a new infant formula must make a submission to FDA, providing required assurances about the formula, at least 90 days before the formula is marketed. Jennewein should be aware that FDA's response to Jennewein's GRAS notice does not alleviate the responsibility of any infant formula manufacturer who intends to market an infant formula that contains 2'-FL to make the submission required by section 412.

Section 301(ll) of the FD&C Act

Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Jennewein's notice that 2'-FL is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing 2'-FL. Accordingly, this response should not be construed to be a statement that foods that contain 2'-FL, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Jennewein, as well as other information available to FDA, the agency has no questions at this time regarding Jennewein's conclusion that 2'-FL is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of 2'-FL. As always, it is the continuing responsibility of Jennewein to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000571, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at **www.fda.gov/grasnoticeinventory (/7993/20171031001515/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)**.

Sincerely,

Dennis M. Keefe, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

(1) Jennewein cites published information confirming the nonpathogenicity and nontoxigenicity of the production strain. Jennewein states that the production strain was also described in GRN 000485 for which FDA had no questions.

More in GRAS Notice Inventory (/7993/20171031001515/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)

Gavin Thompson
Environ International Corporation
1702 E. Highland Ave., Suite 412
Phoenix, AZ 85016

Re: GRAS Notice No. GRN 000571

Dear Dr. Thompson:

The Food and Drug Administration (FDA, we) completed our evaluation of the supplement that you submitted on behalf of Jennewein Biotechnologie, GmgH (Jennewein) to GRN 000571. We received the supplement on July 10, 2019. The supplement addresses a change in the production organism for the production of 2'-fucosyllactose (2'-FL).

We previously responded to GRN 000571 on November 6. 2016. We stated that we had no questions at that time regarding Jennewein's conclusion that that 2'-FL is GRAS for use as an ingredient in non-exempt, milk-based infant formulas for term infants and in toddler formulas at a maximum use level of 2 g/L of reconstituted formula.

In the supplement received July 10, 2019, Jennewein informs us of its view that changing the organism for the production of 2'-FL from the genetically engineered *Escherichia coli* BL21 (DE3) #1540 strain to its parent strain (the genetically engineered *E. coli* BL21 (DE3) #1242 strain) and also including the addition of food-grade lactase at the end of the process if there is excess lactose present at the end of the production run is GRAS, through scientific procedures, for use as an ingredient in non-exempt, milk-based infant formulas for term infants and in toddler formulas at a maximum use level of 2 g/L of reconstituted formula.

Jennewein provided information on the genetic engineering of *E. coli* BL21 (DE3) #1242 in the original submission, GRN 000571. The single difference between strains #1540 and #1242 is a high-temperature expressed lactase used to remove excess lactose from the manufacturing process. In the supplement, Jennewein states that the substitution of extraneously added food-grade lactase will have no effect on the identity and safety of 2'-FL.

Based on the totality of the data and information available, Jennewein concludes that 2'-FL produced using the modified manufacturing process using the progenitor *E. coli* strain #1242 is GRAS for its intended use as an ingredient in non-exempt, milk-based infant formulas for term infants and in toddler formulas at a maximum level of 2 g/L of reconstituted formula.

Potential Labeling Issues

Under section 403(a) of the Federal Food, Drug, and Cosmetic Act (FD&C Act), a food is misbranded if its labeling is false or misleading in any way. Section 403(r) of the FD&C Act lays out the statutory framework for labeling claims characterizing a nutrient level in a food or the relationship of a nutrient to a disease or health-related condition (also referred to as nutrient content claims and health claims). If products containing 2'-FL bear any nutrient content or health claims on the label or in labeling, such claims are subject to the applicable requirements and are under the purview of the Office of Nutrition and Food Labeling (ONFL) in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety did not consult with ONFL on this issue or evaluate any information in terms of labeling claims. Questions related to food labeling should be directed to ONFL.

Intended Use in Infant Formula

Under section 412 of the FD&C Act, a manufacturer of a new infant formula must make a submission to FDA providing required assurances about the formula at least 90 days before the formula is marketed. Our response to Jennewein's supplement does not alleviate the responsibility of any infant formula manufacturer that intends to market an infant formula containing 2'-FL to make the submission required by section 412. Infant formulas are the purview of ONFL.

Section 301(ll) of FD&C Act

Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In our evaluation of Jennewein's supplement concluding that 2'-FL is GRAS under its intended conditions of use, we did not consider whether section 301(ll) or any of its exemptions apply to foods containing 2'-FL. Accordingly, our response should not be construed to be a statement that foods containing 2'-FL, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information that Jennewein provided, as well as other information available to FDA, we have no questions at this time regarding Jennewein's conclusion that 2'-FL is GRAS under its intended conditions of use. This letter is not an affirmation that 2'-FL is GRAS under 21 CFR 170.35. Unless noted above, our review did not address other provisions of the FD&C Act. Food ingredient manufacturers and food producers are responsible for ensuring that marketed products are safe and compliant with all applicable legal and regulatory requirements.

In accordance with 21 CFR 170.275(b)(2), the text of this letter responding to the supplement to GRN 000571 is accessible to the public at www.fda.gov/grasnoticeinventory.

Sincerely,

Susan J. Carlson -S

Digitally signed by Susan J. Carlson -S
Date: 2019.11.08 13:53:50 -05'00'

Susan Carlson, Ph.D.
Director
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

APPENDIX B
AGENCY RESPONSE LETTERS: GRN 571

Agency Response Letter GRAS Notice No. GRN 000571

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CFSAN/Office of Food Additive Safety

November 6, 2015

Gavin Thompson, Ph.D.
Ramboll Environ
2111 East Highland Ave., Suite 402
Phoenix, AZ 85016

Re: GRAS Notice No. GRN 000571

Dear Dr. Thompson:

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(bw)/d to the test groups, and 6000 mg fructo-oligosaccharide/kg bw/d to the reference control group was well tolerated and did not elicit any treatment-related, toxicologically relevant effects. Additionally, Jennewein discusses a published 3-week dietary toxicity study conducted in neonatal piglets using its 2'-FL. Jennewein concludes that up to 292 mg 2'-FL/kg bw/d in males, and 299 mg 2'-FL/kg bw/d in females, did not result in any adverse health effects and did not impact piglet growth. Jennewein also discusses an unpublished 90-day dietary toxicity study in rats, an *in vitro* mutagenicity study, and a genotoxicity study conducted using its 2'-FL. The results of the unpublished 90-day toxicity study corroborate the safety conclusions of the published studies. Jennewein also concludes that 2'-FL is non-mutagenic as demonstrated by both the published and unpublished *in vitro* mutagenicity studies. Based on the totality of the data and information discussed in the notice, Jennewein concludes that 2'-FL is GRAS under the intended conditions of use.

Potential Labeling Issues

In describing the intended uses of 2'-FL and in describing the information that Jennewein relies on to conclude that 2'-FL is GRAS under the conditions of its intended use, Jennewein raises a potential issue under the labeling provisions of the Federal Food, Drug, and Cosmetic Act (FD&C Act). Under section 403(a) of the FD&C Act, a food is misbranded if its labeling is false or misleading in any particular. Section 403(r) of the FD&C Act lays out the statutory framework for the use of labeling claims that characterize the level of a nutrient in a food or that characterize the relationship of a nutrient to a disease or health-related condition. If infant formula products that contain 2'-FL bear any claims on the label or in labeling, such claims are the purview of the Office of Nutrition, Labeling, and Dietary Supplements (ONLDS) in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety neither consulted with ONLDS on this labeling issue nor evaluated the information in your notice to determine whether it would support any claims made about 2'-FL on the label or in labeling.

Intended Use in Infant Formula

Under section 412 of the FD&C Act, a manufacturer of a new infant formula must make a submission to FDA, providing required assurances about the formula, at least 90 days before the formula is marketed. Jennewein should be aware that FDA's response to Jennewein's GRAS notice does not alleviate the responsibility of any infant formula manufacturer who intends to market an infant formula that contains 2'-FL to make the submission required by section 412.

Section 301(ll) of the FD&C Act

Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In its review of Jennewein's notice that 2'-FL is GRAS for the intended uses, FDA did not consider whether section 301(ll) or any of its exemptions apply to foods containing 2'-FL. Accordingly, this response should not be construed to be a statement that foods that contain 2'-FL, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information provided by Jennewein, as well as other information available to FDA, the agency has no questions at this time regarding Jennewein's conclusion that 2'-FL is GRAS under the intended conditions of use. The agency has not, however, made its own determination regarding the GRAS status of the subject use of 2'-FL. As always, it is the continuing responsibility of Jennewein to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter responding to GRN 000571, as well as a copy of the information in this notice that conforms to the information in the GRAS exemption claim (proposed 21 CFR 170.36(c)(1)), is available for public review and copying at **www.fda.gov/grasnoticeinventory (/7993/20171031001515/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)**.

Sincerely,

Dennis M. Keefe, Ph.D.
Director
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition

(1) Jennewein cites published information confirming the nonpathogenicity and nontoxigenicity of the production strain. Jennewein states that the production strain was also described in GRN 000485 for which FDA had no questions.

More in GRAS Notice Inventory (/7993/20171031001515/https://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm)

Gavin Thompson
Environ International Corporation
1702 E. Highland Ave., Suite 412
Phoenix, AZ 85016

Re: GRAS Notice No. GRN 000571

Dear Dr. Thompson:

The Food and Drug Administration (FDA, we) completed our evaluation of the supplement that you submitted on behalf of Jennewein Biotechnologie, GmgH (Jennewein) to GRN 000571. We received the supplement on July 10, 2019. The supplement addresses a change in the production organism for the production of 2'-fucosyllactose (2'-FL).

We previously responded to GRN 000571 on November 6. 2016. We stated that we had no questions at that time regarding Jennewein's conclusion that that 2'-FL is GRAS for use as an ingredient in non-exempt, milk-based infant formulas for term infants and in toddler formulas at a maximum use level of 2 g/L of reconstituted formula.

In the supplement received July 10, 2019, Jennewein informs us of its view that changing the organism for the production of 2'-FL from the genetically engineered *Escherichia coli* BL21 (DE3) #1540 strain to its parent strain (the genetically engineered *E. coli* BL21 (DE3) #1242 strain) and also including the addition of food-grade lactase at the end of the process if there is excess lactose present at the end of the production run is GRAS, through scientific procedures, for use as an ingredient in non-exempt, milk-based infant formulas for term infants and in toddler formulas at a maximum use level of 2 g/L of reconstituted formula.

Jennewein provided information on the genetic engineering of *E. coli* BL21 (DE3) #1242 in the original submission, GRN 000571. The single difference between strains #1540 and #1242 is a high-temperature expressed lactase used to remove excess lactose from the manufacturing process. In the supplement, Jennewein states that the substitution of extraneously added food-grade lactase will have no effect on the identity and safety of 2'-FL.

Based on the totality of the data and information available, Jennewein concludes that 2'-FL produced using the modified manufacturing process using the progenitor *E. coli* strain #1242 is GRAS for its intended use as an ingredient in non-exempt, milk-based infant formulas for term infants and in toddler formulas at a maximum level of 2 g/L of reconstituted formula.

Potential Labeling Issues

Under section 403(a) of the Federal Food, Drug, and Cosmetic Act (FD&C Act), a food is misbranded if its labeling is false or misleading in any way. Section 403(r) of the FD&C Act lays out the statutory framework for labeling claims characterizing a nutrient level in a food or the relationship of a nutrient to a disease or health-related condition (also referred to as nutrient content claims and health claims). If products containing 2'-FL bear any nutrient content or health claims on the label or in labeling, such claims are subject to the applicable requirements and are under the purview of the Office of Nutrition and Food Labeling (ONFL) in the Center for Food Safety and Applied Nutrition. The Office of Food Additive Safety did not consult with ONFL on this issue or evaluate any information in terms of labeling claims. Questions related to food labeling should be directed to ONFL.

Intended Use in Infant Formula

Under section 412 of the FD&C Act, a manufacturer of a new infant formula must make a submission to FDA providing required assurances about the formula at least 90 days before the formula is marketed. Our response to Jennewein's supplement does not alleviate the responsibility of any infant formula manufacturer that intends to market an infant formula containing 2'-FL to make the submission required by section 412. Infant formulas are the purview of ONFL.

Section 301(ll) of FD&C Act

Section 301(ll) of the FD&C Act prohibits the introduction or delivery for introduction into interstate commerce of any food that contains a drug approved under section 505 of the FD&C Act, a biological product licensed under section 351 of the Public Health Service Act, or a drug or a biological product for which substantial clinical investigations have been instituted and their existence made public, unless one of the exemptions in section 301(ll)(1)-(4) applies. In our evaluation of Jennewein's supplement concluding that 2'-FL is GRAS under its intended conditions of use, we did not consider whether section 301(ll) or any of its exemptions apply to foods containing 2'-FL. Accordingly, our response should not be construed to be a statement that foods containing 2'-FL, if introduced or delivered for introduction into interstate commerce, would not violate section 301(ll).

Conclusions

Based on the information that Jennewein provided, as well as other information available to FDA, we have no questions at this time regarding Jennewein's conclusion that 2'-FL is GRAS under its intended conditions of use. This letter is not an affirmation that 2'-FL is GRAS under 21 CFR 170.35. Unless noted above, our review did not address other provisions of the FD&C Act. Food ingredient manufacturers and food producers are responsible for ensuring that marketed products are safe and compliant with all applicable legal and regulatory requirements.

In accordance with 21 CFR 170.275(b)(2), the text of this letter responding to the supplement to GRN 000571 is accessible to the public at www.fda.gov/grasnoticeinventory.

Sincerely,

Susan J. Carlson -S

Digitally signed by Susan J. Carlson -S
Date: 2019.11.08 13:53:50 -05'00'

Susan Carlson, Ph.D.
Director
Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety
and Applied Nutrition

APPENDIX C
PROTEIN ANALYSES

APPENDIX C1
SILVER STAINING RESULTS FOR DETECTABLE PROTEIN

This work demonstrates the absence of any detectable protein in five different batches of the product 2'-fucosyllactose produced by Jennewein Biotechnologie GmbH using SDS-PAGE and silver-staining.

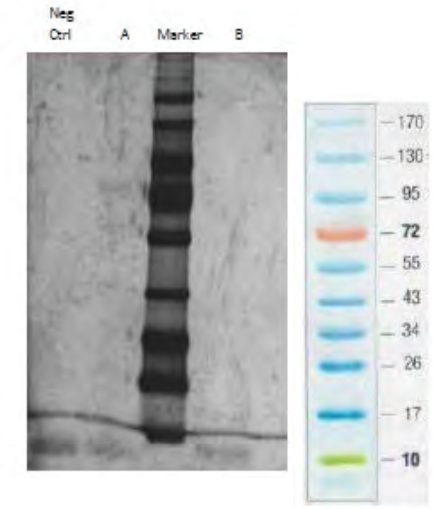


Figure A-1: Silver stained SDS-Gel loaded with Jennewein 2'-Fucosyllactose. From left to right: Negative Control ("Neg Crtl") lane contains Sample Buffer; Lane A contains 16 mg 2'-FL , lot no. L-1001-001500213104618; Marker lane contains PageRuler™ Prestained Protein Ladder, with protein bands corresponding to those shown in to the right of the gel (size given in kDa); Lane B contains 16 mg 2'-FL , lot no. L-1001-1464700033104618.

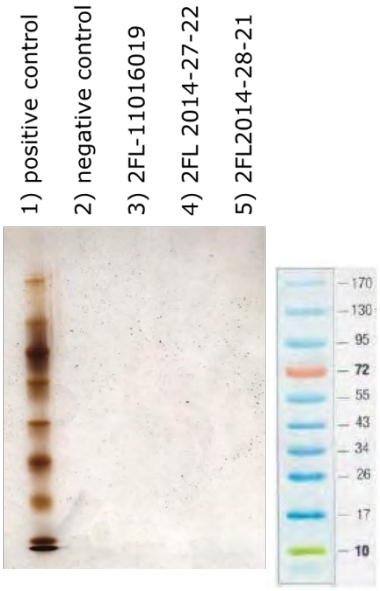


Figure A-2: Silver stained SDS-Gel of three 2'-Fucosyllactose samples (15 mg 2'-FL each) produced by Jennewein Biotechnologie GmbH. From left to right: Lane 1 contains the positive control, PageRuler™ Prestained Protein Ladder, with protein bands corresponding to those shown in to the right of the gel (size given in kDa); Lane 2 contains the negative control, Sample Buffer; Lane 3 contains 16 mg 2'-FL , lot no. 2FL-11016019; Lane 4 contains 16 mg 2'-FL , lot no. 2FL 2014-27-22; Lane 5 contains 16 mg 2'-FL, lot no. 2FL2014-28-21.

The sensitivity of the applied highly sensitive silver-staining method is between 0.2 ng and 0.6 ng. No trace of any protein was detected on the SDS-PAGE (see Figures A-1 and A-2), evidence for the absence of protein in 2'-fucosyllactose produced by Jennewein Biotechnologie GmbH.

APPENDIX C2
ELISA: IMMUNOLOGICALLY ACTIVE CASEIN AND WHEY

Summary

Five lots of Jennewein 2'-fucosyllactose (2'-FL) were evaluated using Enzyme-Linked Immunosorbent Assay (ELISA) for the quantitation of immunologically active casein (IAC) and immunologically active whey (IAW). The limit of quantitation of the assays are 10 ng/mL for the IAC assay, and 30 ng/mL for the IAW assay. Calculating back to the amount of IAC and IAW that could be present in the 2'-FL ingredient (using the higher sample test concentration of 1.1 g/L), the limit of quantitation for the amount of IAC and IAW in the 2'-FL ingredient would be 9.1 mg/kg and 27.3 mg/kg, respectively. Two test concentrations were prepared for each sample: 0.22 g/L and 1.1 g/L.

2'-FL Lot	Test concentration (g/L)	IAC (ng/mL)	IAW (ng/mL)
2FL-2013-50-3434	1.1	27	< LOQ
	0.22	< LOQ	< LOQ
2FL-2013-45-34	1.1	< LOQ	< LOQ
	0.22	< LOQ	< LOQ
2FL-2014-28-21	1.1	< LOQ	< LOQ
	0.22	< LOQ	< LOQ
2FL-2014-27-22	1.1	< LOQ	< LOQ
	0.22	< LOQ	< LOQ
2FL-11016019	1.1	< LOQ	< LOQ
	0.22	< LOQ	< LOQ

Conclusions

No immunological active casein or whey protein was detected at the 0.22 g/L 2'-FL sample preparation concentration. A positive result was obtained in the analysis of one lot of material at the 1.1 g/L sample preparation concentration. This result is likely an artifact of the competitive ELISA tendency to overestimate values in non-matched matrices. However, even if the 27 ppm result at the 1.1 g/L sample preparation concentration is attributable to immunologically active casein, this value corresponds to a very low concentration that is unlikely to result in a reaction in individuals with sensitivity to milk proteins.

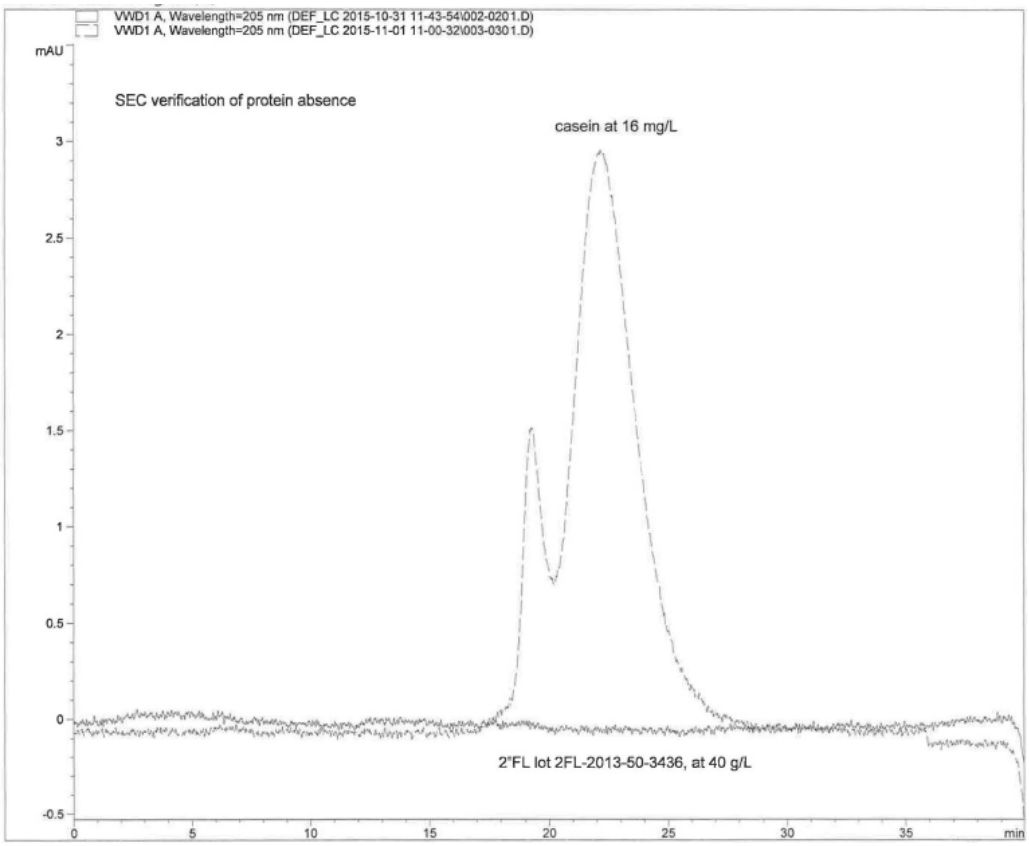
APPENDIX C3
ANALYSIS OF PROTEIN IN 2'-FL USING SIZE EXCLUSION CHROMATOGRAPHY

Summary

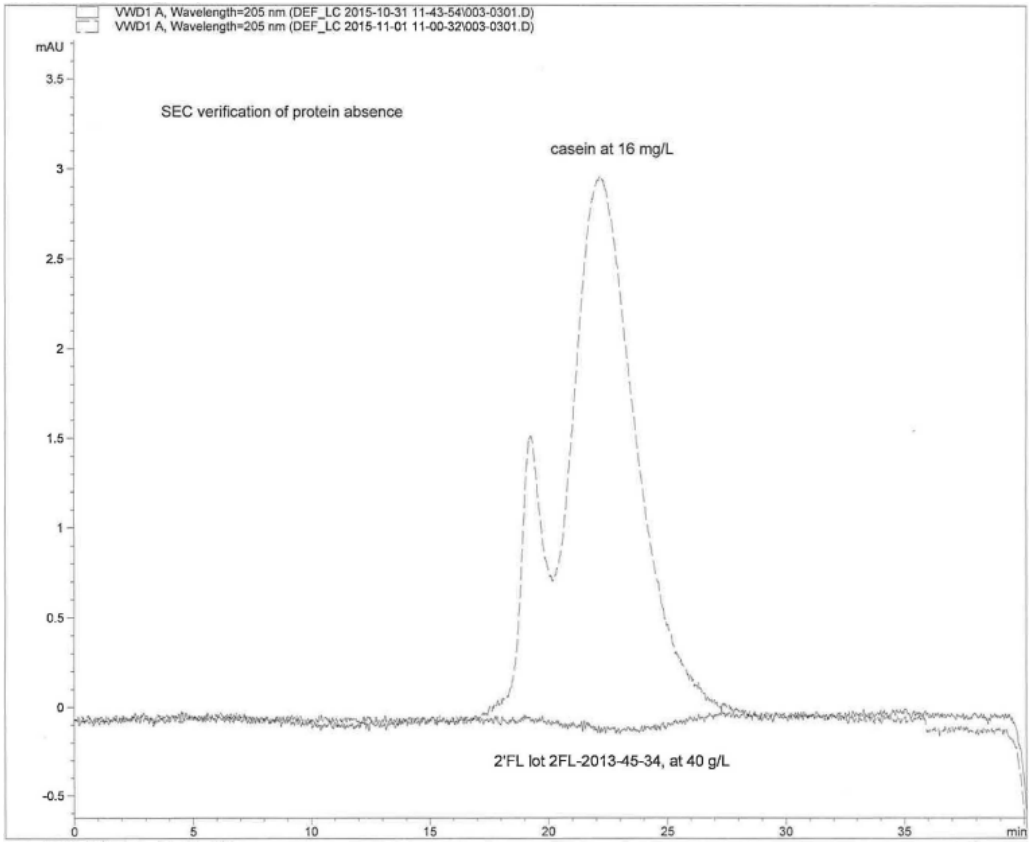
Two lots of 2'-fucosyllactose (2'-FL) from Jennewein Biotechnologie were analyzed for intact protein by size exclusion chromatography, with UV detection at 205 nm. No intact protein was detected in either of the two lots. The method detection limit is 50 micrograms of intact protein per g of 2'-FL.

2'-FL lot	Concentration of intact protein
2FL-2013-50-3436	None detected
2FL-2013-45-34	None detected

Jennewein 2'-FL Lot 2FL-2013-50-3436



Jennewein 2'-FL Lot 2FL-2013-45-34



APPENDIX D
JENNEWEIN 2'-FL: MICRONUCLEUS TEST (CULTURED HUMAN PERIPHERAL LYMPHOCYTES)

LABORATORY of PHARMACOLOGY and TOXICOLOGY

LPT

LPT Report No. 31834

IN VITRO ASSESSMENT OF 2'-FUCOSYLLACTOSE IN THE MICRONUCLEUS TEST IN CULTURED HUMAN PERIPHERAL LYMPHOCYTES

- according to OECD guideline 487 -

Sponsor:

Jennewein Biotechnologie GmbH
Maarweg 32
53616 Rheinbreitbach
Germany

Contact person:

Dr. S. Jennewein

Study conducted by:

LPT Laboratory of Pharmacology and Toxicology GmbH & Co. KG
Redderweg 8
21147 Hamburg
Germany

Contact person:

Dr. phil. J. Leuschner

March 11, 2015

This report consists of 36 pages.

TABLE OF CONTENTS

STATEMENT OF COMPLIANCE

IN VITRO ASSESSMENT OF
2'-FUCOSYLLACTOSE
IN THE MICRONUCLEUS TEST
IN CULTURED HUMAN PERIPHERAL LYMPHOCYTES

- according OECD guideline 487 -

The study was performed in compliance with:

- 'Good Laboratory Practice' Regulations of the EC enacted in Germany in the 'Chemikaliengesetz' [Chemicals Act], current edition;
- 'OECD Principles of Good Laboratory Practice' Document Nos. 1, 8 and 13 ENV/MC/CHEM (98) 17, ENV/JM/MONO (99) 24, ENV/JM/MONO (2002) 9, respectively.

These principles are compatible with 'Good Laboratory Practice' regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA) and Japan (MHLW, MAFF and METI).

There were no deviations from the 'Good Laboratory Practice' regulations. Raw data obtained during the performance of the study are accurately reflected.

[redacted]

Dr. rer. nat. B. Spruth
Study Director

11 Mai 2015

Date

QUALITY ASSURANCE STATEMENT

Based on a quality assurance review, it was concluded that this report accurately reflects the raw data for the study. Methods, procedures and observations are correctly and completely described in the report.

***IN VITRO* ASSESSMENT OF**
2'-FUCOSYLLACTOSE
IN THE MICRONUCLEUS TEST
IN CULTURED HUMAN PERIPHERAL LYMPHOCYTES
- according OECD guideline 487 -

Study Plan dated December 22, 2014.

Date of inspection	Criteria	Date of report to the Study Director and the Management
22 Dec 2014	Study Plan	22 Dec 2014
12 Jan 2015 to 16 Jan 2015	General inspection of micronucleus tests in human peripheral lymphocytes (*in vitro*): seeding cells, preparation and administration of test item, preparation and administration of S9 mix, addition of Cytochalasin B, treatment with KCl, fixing, spotting, staining, evaluation, documentation	16 Jan 2015
11 Mar 2015	Final Report	11 Mar 2015

In addition to the detailed study-based inspections series of routine facility and process-based inspections were also conducted and reported to the management during the course of the study.

Approved and submitted by:

[redacted]

Dr. med. vet. habil. K. R. Sultan
Director of Quality
Assurance Unit (QAU)

11 März 2015
Date

1. SUMMARY

Test samples of 2′-Fucosyllactose were assayed in an *in vitro* micronucleus test using human peripheral lymphocytes both in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix) from Aroclor 1254 induced animals.

The test was carried out employing 2 exposure times without S9 mix: 4 and 20 hours, and 1 exposure time with S9 mix: 4 hours. The harvesting time was 20 hours after the end of exposure. The cytokinesis-block technique was applied.

The test item was completely dissolved in dimethylsulfoxide (DMSO). A correction factor of 1.06 was used as the purity of 2′-Fucosyllactose was 94.1%. Dimethylsulfoxide (DMSO) served as the vehicle control.

The concentrations employed were chosen based on the results of a cytotoxicity study. In this preliminary experiment without and with metabolic activation concentrations of 62.5, 125, 250, 500, 1000, 2000 and 5000 µg 2′-Fucosyllactose/mL medium were employed. No signs of cytotoxicity were noted up to the top concentration of 5000 µg test item/mL medium. 5000 µg/mL is the highest concentration to be tested of no precipitate or limiting cytotoxicity is observed. Hence, 5000 µg/mL were employed as the top concentration for the genotoxicity tests without and with metabolic activation.

No signs of cytotoxicity were noted in the main study in the experiments without and with metabolic activation up to the top concentration of 5000 µg test item/mL medium.

Mitomycin C (at 0.2 µg/mL) and colchicine (at 0.02 µg/mL) were employed as positive controls in the absence and cyclophosphamide (at 20 µg/mL) in the presence of metabolic activation.

Tests without metabolic activation (4- and 20-hour exposure)

The micronucleus frequencies of cultures treated with the concentrations of 500, 1000, 2000 or 5000 µg 2′-Fucosyllactose/mL medium (4-h or 20-h exposure) in the absence of metabolic activation ranged from 1.5 to 4.5 micronuclei per 1000 binucleated cells. There was no dose related increase in micronuclei up to the top concentration of 5000 µg 2′-Fucosyllactose/mL medium. The micronuclei frequency was within the historical control range of the untreated and vehicle control.

Vehicle controls should give reproducibly low and consistent micronuclei frequencies. In this test the following frequencies were observed: vehicle control: 4.0 or 6.5 micronuclei per 1000 binucleated cells for the 4-hour and 20-hour exposure, respectively. The vehicle results were within the historical control ranges.

Test with metabolic activation (4-hour exposure)

The micronucleus frequencies of cultures treated with the concentrations of 500, 1000, 2000 or 5000 µg 2'-Fucosyllactose/mL medium (4-h exposure) in the presence of metabolic activation ranged from 3.0 to 3.5 micronuclei per 1000 binucleated cells. There was no dose related increase in micronuclei up to the top concentration of 5000 µg 2'-Fucosyllactose/mL medium. The micronuclei frequency was within the historical control range of the untreated and vehicle control.

Vehicle controls should give reproducibly low and consistent micronuclei frequencies. In this test a mean frequency of 3.5 micronuclei per 1000 binucleated cells was observed. The vehicle result was within the historical control ranges.

1.1 Conclusion

Under the present test conditions, 2'-Fucosyllactose tested up to a concentration of 5000 µg/mL, in the absence and in the presence of metabolic activation employing two exposure times (without S9) and one exposure time (with S9) revealed no indications of chromosomal damage in the *in vitro* micronucleus test.

In the same test, Mitomycin C and cyclophosphamide induced significant chromosomal damage and colchicine induced significant damage to the cell division apparatus, respectively. Therefore, the test is considered valid.

[redacted]

Dr. rer. nat. B. Spruth
Study Director

11 Mar 2015

Date

2. GENERAL INFORMATION

2.1 Aim of experiment

The *in vitro* micronucleus assay is a genotoxic test system for the detection of chemicals which induce the formation of small membrane bound DNA fragments i.e. micronuclei in the cytoplasm of interphase cells. These micronuclei may originate from acentric fragments (chromosome fragments lacking a centromere) or whole chromosomes which are unable to migrate with the rest of the chromosomes during the anaphase of cell division.

The purpose of the micronucleus assay is to detect those agents which modify chromosome structure and segregation in such a way as to lead to induction of micronuclei in interphase cells.

2.2 Sponsor / Test Facility / Responsible personnel

Sponsor	Jennewein Biotechnologie GmbH Maarweg 32 53616 Rheinbreitbach Germany
Contact person	Dr. Stefan Jennewein Phone: +49 - 2224 989 4501 E-mail: stefan.jennewein@jennewein-biotech.de
Test Facility	**LPT** Laboratory of Pharmacology and Toxicology GmbH & Co. KG Redderweg 8 21147 Hamburg Germany Phone: +49 - 40 - 70 20 20 Fax: +49 - 40 - 70 20 22 60 E-mail: LPT@LPT-Hamburg.de
Study Director / Study conduct	Dr. rer. nat. B. Spruth **LPT,** Redderweg 8 21147 Hamburg Germany
Deputy Study Director	Dr. phil. J. Leuschner
Test Facility Management	Dr. rer. nat. A. Winkler
Statistics	Dipl. Biol. J. Köpcke
Quality Assurance Unit (QAU)	Dr. med. vet. habil. K. R. Sultan
Code number of the	

study in the raw data 31834

2.3 Rules and regulations

The study was carried out in compliance with:

- OECD Guidelines for Testing of Chemicals: In Vitro Mammalian Cell Micronucleus Test (MNvit) (No. 487, Guideline September 26, 2014).

In addition, the 'Good Laboratory Practice' regulations were considered (see the Statement of Compliance and the enclosed GLP Certificate of the Test Facility **LPT**).

Standard Operating Procedures	All work was carried out according to Standard Operating Procedures which were followed for all stages of the study; they may be inspected in those divisions which were engaged in the study and in the Quality Assurance Unit (QAU).
Staff safety	The standard safety precautions operating within the department were applied to this study.

2.4 Archive

Archives of data and specimens	**During the study:** In the depot **LPT,** Redderweg 8 21147 Hamburg Germany **After reporting:** All specimens, retention sample of the test item, written raw data, Study Plan and other documents generated at **LPT** during the course of this study, together with a copy of the final report are stored in the **LPT** archives as required by the German 'Chemikaliengesetz' [Chemicals Act]. The final report will be archived by the Sponsor.
Duration of storage	According to the periods laid down in the German 'Chemikaliengesetz' [Chemicals Act] for at least 15 years; afterwards the Sponsor will be contacted to decide on further use.

2.5 Study dates

Start of study

Date of Study Plan	December 22, 2014
Start of the experimental phase	December 23, 2014
Period of treatment	January 2015

Study termination

Termination of the experimental phase	January 16, 2015
Date of the final report	March 11, 2015

2.6 Study Plan deviations

The study was conducted in accordance with the Study Plan agreed upon. There were no major deviations from this Study Plan. However, the following minor deviations were noted:

- **3.2 Description**: Expiry Date: January 2016 is given in the final report, based on the Certificate of Analysis dated January 08, 2015.
- **4.8 Procedure / Treatment Schedule**: Without S9 mix: (completion due to copy/paste in the Study Plan).

  Two concentrations of colchicine (0.01 or 0.02 µg/mL) were added to each culture of the positive controls.

These minor deviations did not have any effect on the validity and integrity of the scientific results obtained in this study.

3. TEST ITEM

3.1 Identification of the test item

After receipt at **LPT**, the test item was inspected; batch number, amount and characteristics (colour, consistency and form) were determined and compared with information given by the Sponsor. An identification sheet was then filed with the raw data.

Test item	Parameter	LPT Identification	Sponsor Identification
2'-Fucosyllactose	colour consistency form	white solid powder	colourless to ivory solid powder to crystals

No further identification was performed by **LPT** for this study.

3.2 Description

Designation	2'-Fucosyllactose
Batch No.	2FL-2013-43-2632
CAS No.	41263-94-9
Receipt No.	54569
Date of receipt	October 31, 2013
Characterisitcs	White to ivory-coloured powder or crystals
Expiry Date	January 2016
Storage conditions	At room temperature (+10°C to +25°C)
Correction factor	1.06
Purity (HPAEC-PAD)	94.1%
	Further information on the test item is given in Appendix 1 'Certificate of Analysis'
Retention sample of the test item	Stored in **LPT**'s archives.

3.3 Preparation of the test item solution

The test item was completely dissolved in dimethylsulfoxide (DMSO)[1]. A correction factor of 1.06 was used as the purity of 2'-Fucosyllactose was 94.1%. The respective stock solutions were further diluted with DMSO to the appropriate concentrations prior to adding them to the cell culture. Dimethylsulfoxide (DMSO) served as the vehicle control.

Fresh preparations of the test item were used for the treatment in all experimental parts.

[1] Batch no. 4A008607; AppliChem GmbH 64291 Darmstadt, Germany

4. METHODS

4.1 Initial considerations

The test requires the use of an exogenous source of metabolic activation unless the cells are metabolically competent with respect to the substances being tested. The exogenous metabolic activation system does not entirely mimic *in vivo* conditions. Care is also taken to avoid conditions that would lead to artifactual positive results which do not reflect intrinsic genotoxicity, and may arise from such factors as marked changes in pH or osmolality, or by high levels of cytotoxicity.

To analyse the induction of micronuclei, it is essential that mitosis has occurred in both treated and untreated cultures. The most informative stage for scoring micronuclei is in cells that have completed one mitosis during or after treatment with the test item.

4.2 Principle of test method

Cell cultures (human peripheral lymphocytes) were exposed to the test item both with and without metabolic exogenous source of metabolic activation. Concurrent the vehicle dimethylsulfoxide (DMSO) and positive controls were included in all tests. During or after exposure to the test item, the cells were grown for a period sufficient to allow chromosome or spindle damage to lead to the formation of micronuclei in interphase cells. Harvested and stained interphase cells were analysed for the presence of micronuclei. Ideally, micronuclei are only scored in those cells that have completed mitosis during exposure to the test item. This is achieved by scoring only binucleate cells. It is important to demonstrate that cell proliferation has occurred in both the control and treated cultures, and the extent of test item-induced cytotoxicity or cytostasis is assessed in the cultures (or in parallel cultures) that are scored for micronuclei.

4.3 Culture establishment

Human peripheral blood was obtained by venipuncture from young (approximately 18 – 35 years of age), healthy, non-smoking male or female individuals with no known recent exposures to genotoxic chemicals or radiation, and collected in heparinised vessels. Small innocula of whole blood (0.5 mL) were added to tubes containing 5 mL of Chromosome complete culture medium[2] with 1% Penicillin/Streptomycin. The tubes are sealed and incubated at 37°C, and shaken occasionally to prevent clumping.

[2] Biochrom AG, 12247 Berlin, Germany

4.4 Cytokinesis blocker CytoB (Cytochalasin B)

One of the most important considerations in the performance of the assay is ensuring that the cells being scored have completed mitosis during the treatment incubation period. CytoB is the agent that has been most widely used to block cytokinesis because it inhibits actin assembly, and thus prevents separation of daughter cells after mitosis, leading to the formation of binucleated cells. Micronucleus scoring, therefore, can be limited to cells that have gone through mitosis during or after treatment. The effect of the test item on cell proliferation kinetics can be measured simultaneously. The appropriate concentration of CytoB was determined for each cell line to achieve the optimal frequency of binucleated cells in the vehicle control cultures. The appropriate concentration of cytoB is usually between 3 and 6 μg/mL. The concentration used for this assay was 5 μg/mL.

Treatment of cultures with CytoB, and measurement of the relative frequencies of mononucleate, binucleate, and multi-nucleate cells in the culture, provides an accurate method of quantifying the effect on cell proliferation and the cytotoxic or cytostatic activity of a treatment and ensures that only cells that divided during or after treatment are scored.

4.5 Exposure concentrations

2'-Fucosyllactose was completely dissolved in dimethylsulfoxide (DMSO). The vehicle dimethylsulfoxide (DMSO) served as the vehicle control.

The highest concentration should aim to produce 55 ± 5% cytotoxicity. Higher levels may induce chromosome damage as a secondary effect of cytotoxicity. Where cytotoxicity occurs, the test concentrations selected should cover a range from that producing 55 ± 5% cytotoxicity, to little or no cytotoxicity.

If no cytotoxicity or precipitate is observed, the highest test concentration corresponds to 10 mM, 5 mg/mL or 5 μL/mL, whichever is the lowest. The concentrations selected for analysis are, in general, separated by a spacing of no more than $\sqrt{10}$. If the test item exhibit a steep concentration-response curve, it is necessary to more closely space the test item concentrations so that cultures in the moderate and low toxicity ranges also are scored.

When solubility is a limiting factor, the maximum concentration, if not limited by cytotoxicity, would be the lowest concentration at which minimal precipitate is visible in cultures, provided there is no interference with scoring. Evaluation of precipitation would be done by methods such as light microscopy, noting precipitate that persists, or appears during culture (by the end of treatment).

At least three analysable test concentrations were evaluated. In order to achieve this, a preliminary cytotoxicity test was performed to narrow the range of concentrations used for the definitive test. In this preliminary experiment without and with metabolic activation concentrations of 62.5, 125, 250, 500, 1000, 2000 and 5000 µg 2'-Fucosyllactose/mL medium were employed. No signs of cytotoxicity were noted up to the top concentration of 5000 µg test item/mL medium. Hence, 5000 µg/mL were employed as the top concentration for the genotoxicity tests without and with metabolic activation.

4.6 Preparation of S9 mix

Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254, prepared according to MARON and AMES (1983) was purchased from Trinova Biochem[3]. S9 was collected from male rats.

The protein content of the S9 fraction and the cytochrome activity P-450 is presented in Appendix 2 (tested by Moltox[4] and distributed by Trinova Biochem).

The S9 fraction was stored at -80 °C. The S9 mix was freshly prepared on the day of the test according to MARON and AMES (1983): containing the following components (per 15 mL):

- 1.5 mL rat liver S9 (Aroclor 1254-induced)
- 0.3 mL 0.4 M $MgCl_2$ + 1.65 M KCl salt solution (sterile stock solution)
- 21.1 mg glucose-6-phosphate[5] (5.41 mM)
- 45.9 mg NADP[6] (3.89 mM)
- 5.7 mL 20 mM Hepes buffer, pH 7.4 (sterile stock solution)
- 7.5 mL phosphate buffer[7]

Afterwards the S9 mix was filter-sterilised by using a 0.45 µm filter and kept on ice.

4.7 Vehicle and positive controls

Concurrent positive and vehicle controls both with and without metabolic activation were included in each experiment. Positive controls were needed to demonstrate the ability of the used human peripheral lymphocytes and the test protocol, to identify clastogens and aneugens, and to affirm the metabolic capability of the S9 preparation.

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[3] Lot. no. 3305; Trinova Biochem GmbH, 35394 Gießen, Germany

[4] Molecular Toxicology, Inc., Boone, NC 28607, USA

[5] SIGMA, St. Louis, MO, USA

[6] Serva, 82152 Martinsried, Germany

[7] GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany

The positive controls should employ known inducers of micronucleus formation at concentrations expected to give small, but reproducible increases over background, and demonstrate the sensitivity of the test system.

Positive control concentrations are chosen so that the effects are clear but do not immediately reveal the identity of the coded slides to the reader. A clastogen that requires metabolic activation (e.g. cyclophosphamide) was used to demonstrate both the metabolic competence and the ability of the test system to detect clastogens.

At the present time, no aneugens are known that require metabolic activation for their genotoxic activity. Currently accepted positive controls for aneugenic activity are, for example, colchicine and vinblastine.

Vehicle controls were included for every harvest time.

positive controls	without metabolic activation	with metabolic acitvation
clastogen	Mitomycin C[8] (c = 0.1 µg/mL and c = 0.2 µg/mL)	Cyclophosphamide[9] (c = 10 µg/mL and c = 20 µg/mL)
aneugen	Colchicine[10] (c = 0.01 µg/mL) and (c = 0.02 µg/mL)	-

The following concentrations were so far not evaluated, as it was thought that they would provide no further information: 0.1 µg Mitomycin C/mL, 0.01 µg Colchicine/mL in the experiments without S9 mix and 10 µg Cyclophosphamide/mL in the experiment with S9 mix, respectively. The vehicle dimethylsulfoxide (DMSO) served as the vehicle control.

4.8 Procedure / Treatment Schedule

In order to maximise the probability of detecting an aneugen or clastogen acting at a specific stage in the cell cycle, it is important that sufficient numbers of cells are treated with the test item during all stages of their cell cycles. Hence, 0.5 mL of freshly prepared blood and 5 mL of Chromosome complete culture medium 1A with Phytohemagglutinin and 1% Penicillin/Streptomycin were seeded with an appropriate concentration of the test item in DMSO for each target concentration of the test item in the test medium and each experiment. Any possible test item precipitation was checked before and after each experiment. Evaluation of precipitation was done by

[8] Batch no. SLBH9906V; SIGMA-ALDRICH Chemie GmbH, 82024 Taufkirchen, Germany

[9] Batch no. SLBC0666V; SIGMA-ALDRICH Chemie GmbH, 82024 Taufkirchen, Germany

[10] Batch no. 3668; Carl Roth GmbH & Co. KG, 76185 Karlsruhe, Germany

light microscopy at the beginning and end of treatment. Theoretical considerations, together with published data, indicate that most aneugens and clastogens are detected by a short term treatment period of 4 hours in the presence and absence of S9, followed by removal of the test item and a growth period of 1.5 cell cycles. Cells were sampled at a time equivalent to about 1.5 times the normal (i.e. untreated) cell cycle length either after the beginning or at the end of treatment. Sampling or recovery times would have been extended if it is known or suspected that the test item affects the cell cycling time (e.g. when testing nucleoside analogues). Because of the potential cytotoxicity of S9 preparations for cultured mammalian cells, an extended exposure treatment was used only in the absence of S9.

All treatments commenced and ended while the cells were growing exponentially.

Cell treatment and harvest times for the used human lymphocytes line:

Without S9 mix:

4-hours exposure:

Cultures were initiated and maintained as described in Section 4.3. After 48 hours the medium was replaced by 4.95 mL of fresh Ham's F10 medium[11] with fetal calf serum (FCS)[11]. Two concentrations of Mitomycin C (0.1 or 0.2 µg/mL) and colchicine (0.01 or 0.02 µg/mL) were added to each culture of the positive controls. Vehicle controls, test item treatments, and positive controls were added at a volume of 50 µL to obtain the corresponding target concentrations. The cultures were then incubated for 4 hours at +37°C. Afterwards the medium was removed and the cultures were washed twice with Ham's F10 medium. After addition of 5 mL Chromosome medium containing 5 µg/mL Cytochalasin B the cultures were incubated for further 20 hours at 37°C.

20-hours exposure:

Cultures were initiated and maintained as described in Section 4.3. After 48 hours the medium was replaced by 4.95 mL of fresh Ham's F10 medium with FCS. Two concentrations of Mitomycin C (0.1 or 0.2 µg/mL) and colchicine (0.01 or 0.02 µg/mL) were added to each culture of the positive controls. Vehicle controls, test item treatments, and positive controls were added at a volume of 50 µL to obtain the corresponding target concentrations. The cultures were then incubated for 20 hours at +37°C. Afterwards the medium was removed and the cultures were washed twice with Ham's F10 medium. After addition of 5 mL Chromosome medium containing 5 µg/mL Cytochalasin B the cultures were incubated for further 20 hours at 37°C.

[11] GIBCO Invitrogen GmbH, Technologiepark Karlsruhe, 76131 Karlsruhe, Germany

With S9 mix:

4-hours exposure:

Cultures were initiated and maintained as described in Section 4.3. After 48 hours the medium was carefully removed and replaced by 4.45 mL Ham's F10 medium with FCS and 0.5 mL S9 Mix. Two concentrations of cyclophosphamide (10 or 20 µg/mL) were added to each culture of the positive control. Vehicle control, test item treatments, and positive control were added at a volume of 50 µL to obtain the corresponding target concentrations. The cultures were then incubated for 4 hours at +37°C. Afterwards the medium was removed and the cultures were washed twice with Ham's F10 medium. After addition of 5 mL Chromosome medium containing 5 µg/mL Cytochalasin B the cultures were incubated for further 20 hours at 37°C.

Experiment 1:

Hours	**Absence of S9** **4-h exposure / 20-h sampling**	**Presence of S9** **4-h exposure / 20-h sampling**
0	Commence treatment	Commence treatment
+ 4	Remove treatment medium, wash and add fresh medium, add CytoB	Remove treatment medium, wash and add fresh medium, add CytoB
+ 20	Harvest, prepare slides	Harvest, prepare slides

In a second experiment a 20-h continuous treatment without metabolic activation was conducted.

Experiment 2:

Hours	**Absence of S9** **20-h exposure / 20-h sampling**
0	Commence treatment
+20	Remove treatment medium, wash and add fresh medium, add CytoB
+ 20	Harvest, prepare slides

Duplicate cultures were used for each test item concentration and for the vehicle and positive control cultures.

4.9 Culture harvesting and slide preparation

Each culture was harvested and processed separately. After the test item incubation, mitotic activity was arrested by the addition of CytoB to each culture at a final concentration of 5 µg/mL. After an additional incubation of 20 hours the cultures were centrifuged for 10 minutes at 800 rpm, the supernatant was discarded and the cells resuspended in KCl (1.65 M). After incubation for 17 minutes at 37°C, the cell

suspensions were centrifuged for 10 minutes at 800 rpm. The supernatant was discarded and 5 mL of freshly prepared fixative (3 parts methanol : 1 part glacial acetic acid v/v) added. The cells were left in fixative for 30 minutes followed by centrifugation at 800 rpm. The supernatant was carefully removed and discarded, and the cell pellet was resuspended in about 0.5 mL of fresh fixative and 30% glacial acetic acid by repeated aspiration through a Pasteur pipette. Two drops of this cell suspension were dropped onto a pre-warmed, pre-cleaned microscope slide.
The slides were left to air-dry at room temperature and were then stained using 10% Giemsa.

4.10 pH values and osmolality measurements

The pH and osmolality of the negative control and all test item formulations in the medium were determined for each experiment employing the methods given below:

pH values: using a digital pH meter type pH 540 WTW,
Osmolality: with a semi-micro osmometer[12].

[12] KNAUER, 14163 Berlin, Gemany

5. ANALYSIS

1000 binucleated cells per duplicate cell culture were scored to assess the frequency of cells with one, two, or more than two micronuclei. Additionally, the cells were 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 cell cycles per cell during the period of exposure to CytoB, and is used to calculate cell proliferation.

The RI indicates the relative number of nuclei in treated cultures compared to control cultures and can be used to calculate the % cytostasis:

$$CBPI = \frac{((\text{No. mononucleate cells}) + (2 \times \text{No. binucleate cells}) + (3 \times \text{No. multinucleate cells}))}{(\text{Total number of cells})}$$

Thus, a CBPI of 1 (all cells are mononucleate) is equivalent to 100% cytostasis.

Cytostasis = 100 - RI

$$RI = \frac{((\text{No. binucleated cells}) + (2 \times \text{No. multinucleate cells})) \div (\text{Total number of cells})_T}{((\text{No. binucleated cells}) + (2 \times \text{No. multinucleate cells})) \div (\text{Total number of cells})_C} \times 100$$

T = treated cultures
C = control cultures

Thus, an RI of 53% means that, compared to the numbers of cells that have divided to form binucleate and multinucleate cells in the control culture, only 53% of this number divided in the treated culture, i.e. 47% cytostasis.

All slides, including those of the solvent controls, were independently coded before the microscopic analysis.

The micronucleus frequencies were analysed in at least 2000 binucleated cells per concentration (at least 1000 binucleated cells per culture; two cultures per concentration). If substantially fewer than 1000 binucleate cells per culture are available for scoring at each concentration, and if a significant increase in micronuclei is not detected, the test would be repeated using more cells, or at less toxic concentrations, whichever is appropriate. Care was taken not to score binucleate cells with irregular shapes or where the two nuclei differ greatly in size; neither would binucleate cells be confused with poorly spread multi-nucleate cells. Cells containing more than two main nuclei were not analysed for micronuclei, as the baseline micronucleus frequency might be higher in these cells. Scoring of mononucleate cells is acceptable if the test item is shown to interfere with CytoB activity.

6. ACCEPTABILITY CRITERIA

The assay demonstrates its ability to reliably and accurately detect substances of known aneugenic and clastogenic activity, with and without metabolic activation.

Vehicle control and untreated cultures give reproducibly low and consistent micronuclei frequencies. Data from vehicle and positive controls are used to establish historical control ranges (see Appendix 3). These values are used in deciding the adequacy of the concurrent vehicle controls or positive controls for an experiment.

7. STATISTICAL EVALUATION AND INTERPRETATION OF RESULTS

Only the frequencies of binucleate cells with micronuclei (independent of the number of micronuclei per cell) were used in the evaluation of micronucleus induction. Concurrent measures of cytotoxicity and/or cytostasis for all treated and vehicle control cultures were determined. Individual culture data were provided.

If a test item induces a concentration-related increase or a statistical significant and reproducible increase in the number of cells containing micronuclei, it is classified as a positive result.

Consideration of whether the observed values are within or outside of the historical control range can provide guidance when evaluating the biological significance of the response.

The assessment was carried out by a comparison of the samples with the positive and the vehicle control, using a chi-square test corrected for continuity according to YATES (COLQUHOUN, 1971[1]) as recommended by the UKEMS guidelines (The United Kingdom Branch of the European Environmental Mutagen Society: Report of the UKEMS subcommittee on guidelines for mutagenicity testing, part III, 1989: Statistical evaluation of mutagenicity data).

A positive result from the *in vitro* micronucleus test indicates that the test item induces chromosome damage or damage to the cell division apparatus.

Negative results indicate that, under the test conditions used, the test substance does not induce chromosome breaks and/or gain or loss in cultured mammalian cells.

There is no requirement for verification by additional testing of a clear positive or negative response.

Equivocal results may be clarified by analysis of another 1000 cells from all the cultures to avoid loss of blinding. If this approach does not resolve the result, further testing would be necessary. Modification of study parameters over an extended or narrowed range of conditions, as appropriate, would be considered in follow-up experiments. Study parameters that might be modified include the test concentration spacing, the timing of treatment and cell harvest, and/or the metabolic activation conditions.

Although most experiments give clearly positive or negative results, in some cases the data set would preclude making a definite judgement about the activity of the test item. These equivocal or questionable responses may occur regardless of the number of times the experiment is repeated.

8. REFERENCES

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9. RESULTS AND DISCUSSION

The concentrations employed were chosen based on the results of a cytotoxicity study. In this preliminary experiment without and with metabolic activation concentrations of 62.5, 125, 250, 500, 1000, 2000 and 5000 µg 2'-Fucosyllactose/mL medium were employed. No signs of cytotoxicity were noted up to the top concentration of 5000 µg test item/mL medium (see table 1). 5000 µg/mL is the highest concentration to be tested of no precipitate or limiting cytotoxicity is observed. Hence, 5000 µg/mL were employed as the top concentration for the genotoxicity tests without and with metabolic activation.

No signs of cytotoxicity were noted in the main study in the experiments without and with metabolic activation up to the top concentration of 5000 µg test item/mL medium.

Mitomycin C (at 0.2 µg/mL) and colchicine (at 0.02 µg/mL) were employed as positive controls in the absence and cyclophosphamide (at 20 µg/mL) in the presence of metabolic activation.

Tests without metabolic activation (4- and 20-hour exposure)

The micronucleus frequencies of cultures treated with the concentrations of 500, 1000, 2000 or 5000 µg 2'-Fucosyllactose/mL medium (4-h or 20-h exposure) in the absence of metabolic activation ranged from 1.5 to 4.5 micronuclei per 1000 binucleated cells. There was no dose related increase in micronuclei up to the top concentration of 5000 µg 2'-Fucosyllactose/mL medium. The micronuclei frequency was within the historical control range of the untreated and vehicle control.

Vehicle controls should give reproducibly low and consistent micronuclei frequencies. In this test the following frequencies were observed: vehicle control: 4.0 or 6.5 micronuclei per 1000 binucleated cells for the 4-hour and 20-hour exposure, respectively. The vehicle results were within the historical control ranges.

Test with metabolic activation (4-hour exposure)

The micronucleus frequencies of cultures treated with the concentrations of 500, 1000, 2000 or 5000 µg 2'-Fucosyllactose/mL medium (4-h exposure) in the presence of metabolic activation ranged from 3.0 to 3.5 micronuclei per 1000 binucleated cells. There was no dose related increase in micronuclei up to the top concentration of 5000 µg 2'-Fucosyllactose/mL medium. The micronuclei frequency was within the historical control range of the untreated and vehicle control.

Vehicle controls should give reproducibly low and consistent micronuclei frequencies. In this test a mean frequency of 3.5 micronuclei per 1000 binucleated cells was observed. The vehicle result was within the historical control ranges. For details see Appendix 3.

No test item-related increase in micronucleus frequencies (significant at $p \leq 0.05$) was noted and no dose-response relationship was noted.
In the same test, Mitomycin C and cyclophosphamide induced significant chromosomal damage and colchicine induced significant damage to the cell division apparatus, respectively. Therefore, the test is considered valid.

A summary of the results (means of the duplicate cultures) of this study are listed in tables 2 and 3. Individual values of the experiment without metabolic activation (4- and 20-hour exposure) can be taken from tables 4 (4-hour) and 5 (20-hour exposure). Individual values of the experiments with metabolic activation (4-hour exposure) can be taken from table 6.

The following pH and osmolality data of the vehicle control and of all test item formulations in the medium were determined:

Concentration of 2'-Fucosyllactose in the exposure medium [µg/mL]	**pH value**	**osmolality [mOsmol/kg]**
medium	7.83	295.0
0, vehicle control	7.98	435.0
250	7.98	431.0
500	7.97	430.0
1000	7.95	435.0
2000	7.93	430.0
5000	7.94	420.0

No relevant changes in pH or osmolality of the formulations were noted.

TABLE 1 Estimation of the cytotoxicity

Concentration of 2'-Fucosyllactose [µg/mL medium]	S9 mix	Number of mononucleate cells per 1000 cells scored	binucleate	multinucleate	CBPI	RI [%]
		4-hour exposure				
0	+	240	662	98	1.86	100
62.5	+	210	712	78	1.87	101
125	+	256	648	96	1.84	98
250	+	228	651	121	1.89	103
500	+	284	626	90	1.81	94
1000	+	216	668	116	1.90	105
2000	+	246	676	78	1.83	97
5000	+	202	644	154	1.95	110
		20-hour exposure				
0	-	204	613	183	1.98	100
62.5	-	186	686	128	1.94	96
125	-	191	672	137	1.95	97
250	-	158	660	182	2.02	104
500	-	250	620	130	1.88	90
1000	-	212	610	178	1.97	99
2000	-	198	654	148	1.95	97
5000	-	170	698	132	1.96	98

CBPI Cytokinesis block proliferation index
RI Replicative Index
\+ with metabolic activation
\- without metabolic activation

Summary tables

Table 2 Experiments without metabolic activation (S9 mix)

1st experiment					2nd experiment				
4-h exposure					20-h exposure				
Concentration [µg/mL medium]	CBPI	RI [%]	Number of binucleated cells scored	Number of micronuclei per 1000 binucleated cells	Concentration [µg/mL medium]	CBPI	RI [%]	Number of binucleated cells scored	Number of micronuclei per 1000 binucleated cells
DMSO					DMSO				
0	1.82	100	2000	4.0	0	1.77	100	2000	6.5
2'-Fucosyllactose					2'-Fucosyllactose				
500	1.81	99	2000	3.5	500	1.59	77	2000	3.5
1000	1.85	104	2000	4.5	1000	1.68	89	2000	4.0
2000	1.77	93	2000	4.0	2000	1.76	99	2000	4.0
5000	1.77	94	2000	2.0	5000	1.62	81	2000	1.5
Colchicine					Colchicine				
0.02	1.79	97	2000	28.5 s.	0.02	1.59	77	2000	21.0 s.
Mitomycin C					Mitomycin C				
0.2	1.61	75	2000	26.0 s.	0.2	1.61	80	2000	42.0 s.

CBPI = Cytokinesis block proliferation index
RI = Replicative Index
s. = significantly different from negative control ($p \leq 0.05$)

Summary tables (continued)

Table 3 Experiment with metabolic activation (S9 mix)

Concentration [µg/mL medium]	CBPI	RI [%]	Number of binucleated cells scored	Number of micronuclei per 1000 binucleated cells
		4-h exposure		
DMSO				
0	1.84	100	2000	3.5
2'-Fucosyllactose				
500	1.82	98	2000	3.0
1000	1.85	102	2000	3.5
2000	1.88	105	2000	3.0
5000	1.81	96	2000	3.0
Cyclophosphamide				
20	1.52	62	2000	22.5 s.

CBPI = Cytokinesis block proliferation index
RI = Replicative Index
s. = significantly different from negative control ($p \leq 0.05$)

Individual data

Table 4 Experiment without metabolic activation (S9 mix) - 4-h exposure

Culture number	Concentration [µg/mL medium]	Number of mononucleate cells per 1000 cells scored	Number of binucleate cells per 1000 cells scored	Number of multinucleate cells per 1000 cells scored	CBPI	RI [%]	Number of binucleated cells scored	Number of micronuclei per 1000 binucleated cells	Significance chi^2-test
DMSO									
1	0	204	732	64	1.86	100	1000	4	-
11	0	262	698	40	1.78	100	1000	4	-
2'-Fucosyllactose									
5	500	255	705	40	1.79	92	1000	4	n.s.
15	500	256	668	76	1.82	105	1000	3	n.s.
4	1000	256	694	50	1.79	92	1000	4	n.s.
14	1000	206	689	105	1.90	115	1000	5	n.s.
3	2000	257	667	76	1.82	95	1000	5	n.s.
13	2000	318	658	24	1.71	91	1000	3	n.s.
2	5000	342	627	31	1.69	80	1000	2	n.s.
12	5000	224	708	68	1.84	108	1000	2	n.s.
Colchicine									
9	0.02	280	697	23	1.74	86	1000	31	s.
19	0.02	262	635	103	1.84	108	1000	26	s.
Mitomycin C									
7	0.2	478	498	24	1.55	64	1000	28	s.
17	0.2	354	636	10	1.66	85	1000	24	s.

n.s. = not significantly different from negative control ($p \leq 0.05$)
s. = significantly different from negative control ($p \leq 0.05$)
CBPI = Cytokinesis block proliferation index
RI = Replicative Index

Individual data

Table 5 Experiment without metabolic activation (S9 mix) - 20-h exposure

Culture number	Concentration [µg/mL medium]	Number of mononucleate	binucleate cells per 1000 cells scored	multinucleate	CBPI	RI [%]	Number of binucleated cells scored	Number of micronuclei per 1000 binucleated cells	Significance chi^2-test
DMSO									
1	0	371	495	134	1.76	100	1000	8	-
11	0	334	564	102	1.77	100	1000	5	-
2'-Fucosyllactose									
5	500	451	498	51	1.60	79	1000	2	n.s.
15	500	464	495	41	1.58	75	1000	5	n.s.
4	1000	344	596	60	1.72	95	1000	3	n.s.
14	1000	411	534	55	1.64	83	1000	5	n.s.
3	2000	292	599	109	1.82	108	1000	3	n.s.
13	2000	378	554	68	1.69	90	1000	5	n.s.
2	5000	453	483	64	1.61	80	1000	2	n.s.
12	5000	436	502	62	1.63	82	1000	1	n.s.
Colchicine									
9	0.02	421	537	42	1.62	82	1000	23	s.
19	0.02	486	483	31	1.55	71	1000	19	s.
Mitomycin C									
7	0.2	494	473	33	1.54	71	1000	52	s.
17	0.2	355	610	35	1.68	88	1000	32	s.

n.s. = not significantly different from negative control ($p \leq 0.05$)
s. = significantly different from negative control ($p \leq 0.05$)
CBPI = Cytokinesis block proliferation index
RI = Replicative Index

Individual data

Table 6 First experiment with metabolic activation (+S9 mix) - 4-h exposure

Culture number	Concentration [µg/mL medium]	Number of mononucleate cells per 1000 cells scored	Number of binucleate cells per 1000 cells scored	Number of multinucleate cells per 1000 cells scored	CBPI	RI [%]	Number of binucleated cells scored	Number of micronuclei per 1000 binucleated cells	Significance chi^2-test
DMSO									
1	0	201	734	65	1.86	100	1000	3	-
9	0	252	678	70	1.82	100	1000	4	-
2'-Fucosyllactose									
5	500	194	746	60	1.87	101	1000	4	n.s.
13	500	285	659	56	1.77	94	1000	2	n.s.
4	1000	228	740	32	1.80	93	1000	4	n.s.
12	1000	232	636	132	1.90	110	1000	3	n.s.
3	2000	281	613	106	1.83	97	1000	2	n.s.
11	2000	186	708	106	1.92	112	1000	4	n.s.
2	5000	222	710	68	1.85	99	1000	4	n.s.
10	5000	311	623	66	1.76	93	1000	2	n.s.
Cyclophosphamide									
7	20	456	528	16	1.56	65	1000	21	s.
15	20	532	456	12	1.48	59	1000	24	s.

n.s. = not significantly different from negative control ($p \leq 0.05$)
s. = significantly different from negative control ($p \leq 0.05$)
CBPI = Cytokinesis block proliferation index
RI = Replicative Index

A P P E N D I X 1

Certificate of Analysis

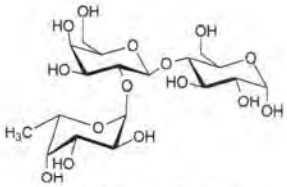
Jennewein
Biotechnologie GmbH

Version 1.5 / Oktober 2013

Analysenzertifikat / Certificate of Analysis

2'-Fucosyllactose

1) Bezeichnung des Stoffs und Zusammensetzung / Product description and composition

Weitere Produktnamen / Synonyms	2'-FL 2'-fucosyl-D-lactose Fuc-α-1,2-Gal-β-1,4-Glc α-L-Fuc-(1->2)-β-D-Gal-(1->4)-D-Glc
Formel / Formula	$C_{18}H_{32}O_{15}$
Strukturformel / Chemical structure	 2'-Fucosyllactose
Molekulargewicht / Molecular weight	488,44 g/mol
CAS-Nr. / CAS no.	41263-94-9

2) Analyse / Analysis

Methode / Analytical Test	**Spezifikation / Specification**	**Ergebnis / Results**
Lot-Nr. / Lot No.		2FL-2013-43-2632
Erscheinungsbild (Farbe) / Appearance (Colour)	farblos bis elfenbeinfarben / white to ivory-coloured	entspricht / corresponds
Erscheinungsbild (Form) / Appearance (Form)	Pulver bis Kristalle / powder to crystals	entspricht / corresponds
Reinheit (HPAEC-PAD) / Purity (HPAEC-PAD)		94.1 %
In Spuren enthalten / Contains traces of		3-Fucosyllactose, Difucosyllactose, Lactose, Fucosylgalactose
Wassergehalt / Water Content		5.2 %
Erscheinigungsbild in Lösung / Appearance in solution	klar, farblos bis leicht gelblich / clear, colourless to slightly yellow	entspricht / corresponds
Verfallsdatum / Date of expiry	**Mindestens haltbar bis** / Best before: 01/2016	

Achtung! Produkt ist extrem hygroskopisch! Bitte trocken und unter Luftausschluss lagern!
Caution! Product is extremely hygroscopic! Please store dry and in an airtight container!

Unterschrift / Signature:

Rheinbreitbach, 08.01.2015
Datum / Date

[redacted]
Qualitätssicherung / Quality Control Manager

A P P E N D I X 2

Post-mitochondrial fraction (S9 fraction)

TRINOVA Biochem GmbH

MOLTOX®
Molecular Toxicology, Inc.

POST MITOCHONDRIAL SUPERNATANT (S9)
QUALITY CONTROL & PRODUCTION CERTIFICATE

Animal Information	**Part Number Information**	
SPECIES: Rat	**LOT NO.:** 3305	**PREP:** July 16, 2014
STRAIN: Sprague Dawley	**PART NO.:** 11-101	**EXPIRY:** July 16, 2016
SEX: Male	**VOLUME:** 5 mL	**INDUCING AGENT:** Aroclor 1254, (Monsanto KL615), 500 mg/kg i.p.
AGE: 5 – 6 weeks	**BUFFER:** 0.15 M KCl	
WEIGHT: 175 – 199 g	**STORAGE:** At or below -70°C	
TISSUE: Liver		

REFERENCE: Maron, D & Ames, B., *Mutat Res*, **113**: 173, 1983. **For Research Purposes Only**

BIOCHEMISTRY:

- PROTEIN: 42.3 mg/ml — Assayed according to the method of Lowry et al., *JBC* **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction	
BROD	2B1, 2B2	139.3	Assays for ethoxyresorufin-0-dethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 72.8, 56.5, 29.9, & 32.3 for BROD, EROD, MROD and PROD, respectively.
EROD	1A1, 1A2	199.7	
MROD	1A1, 1A2	118.6	
PROD	2B1, 2B2	58.4	

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Duplicate plates were read after 40 - 48 h incubation at 35 ± 2°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION — The ability of the sample to activate ethidium (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**: 299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

No. His+ Revertants	
TA98	TA1535
307.6	1674

Dilutions of the sample S9, ranging from 0.2 – 10% in S9 mix, were tested for their ability to activate benzo(α)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted as described by Maron & Ames, (*Mutat Res* **113**: 173, 1983).

	µl S9 per plate/number *his*⁺ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg))	95	186	321	474	799	985
2-AA (2.5 µg)	108	411	2085	2479	2748	1639

Approved: [redacted] 07/21/14

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AEO

A P P E N D I X 3

Historical Background data *in vitro* Micronucleus Test in cultured human peripheral lymphocytes

The micronucleus frequencies of the vehicle controls without and with metabolic activation for the last 13 studies (most recent background data, not audited by the QAU-department) are given as follows:

	Micronucleus frequency per 1000 cells			
	Without metabolic activation (4-h or 20-h exposure)		**With metabolic activation (4-h exposure**	
	Untreated control	**Vehicle control**	**Untreated control**	**Vehicle control**
mean	6.6	6.7	6.4	6.3
SD	2.9	3.0	2.6	4.2
range	2.0 - 17	3.0 - 18	3.0 - 13	1.0 - 22
	Mitomycin C Positive control	**Colchicine Positive control**	**Cyclophosphamide Positive control**	
mean	47.8	25.9	44.8	
SD	36.0	9.8	38.1	
range	17 - 137	15 - 63	14 - 158	

SD = Standard deviation

No further untreated negative controls (lacking solvent/vehicle) were used in this test, as the laboratory historical control data demonstrate that no genotoxic or other deleterious effects are induced by the chosen solvent at the concentrations used, as required by the guideline.

A P P E N D I X 4

GLP Certificate of the Test Facility LPT

FREIE UND HANSESTADT HAMBURG
Behörde für Gesundheit und Verbraucherschutz

Gute Laborpraxis / Good Laboratory Practice

GLP – Bescheinigung / Statement of GLP Compliance

(gemäß/according to § 19b Abs.1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/ EG wurde durchgeführt in:

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/ EEC at:

[X] Prüfeinrichtung/Test facility [ ] Prüfstandort/ Test site

LPT Laboratory of Pharmacology and Toxicology GmbH & Co. KG
Redderweg 8
21147 Hamburg
(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and address)

Prüfungen nach Kategorien/ Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

Kategorie 2, 3, 4 und 9 (Sicherheitspharmakologie und Auftragsarchiv)

Datum der Inspektion/ Date of Inspection:
(Tag.Monat.Jahr/day.month.year)

16., 17., 18. und 19.04.2013

Die/Der genannte Prüfeinrichtung /Prüfstandort befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.
Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung/diesem Prüfstandort die oben genannten Prüfungen unter Einhaltung der GLP-Grundsätze durchgeführt werden können.

The above mentioned test facility/test site is included in the national GLP Compliance Programme and is inspected on a regular basis.

Based on the inspection report it can be confirmed, that this test facility/ test site is able to conduct the aforementioned studies in compliance with the Principles of GLP

Hamburg, den 14.05.2014

Amt für Arbeitsschutz - Arbeitnehmerschutz * Hamburg *

Dr. Volker Kregel
Direktor des Amtes für Arbeitsschutz

Behörde für Gesundheit und Verbraucherschutz
Marckmannstraße 129b, 20539 Hamburg

APPENDIX E

PREVIOUSLY REVIEWED SAFETY STUDIES IN GRN 000571 AND SUPPLEMENT 1 TO GRN 000571

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species / Sex / Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
Genotoxicity / Mutagenicity						
OECD 471	*S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537)	up to 5000 µg/plate; with & without metabolic activation	No cytotoxicity; no increase in revertant colonies; no mutagenic effects.	Coulet *et al.* 2013	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.	GRN 000571
Mutagenicity OECD 471	*S. typhimurium* (TA1535, TA1537, TA98, and TA100); *E. coli* strain WP2 *uvrA*	Up to 5000 µg/plate with & without metabolic activation	No cytotoxicity to any of the strains tested; no significant or dose related increase in revertant colonies; no mutagenic effect.	Verspeek-Rip *et al.* 2015 as cited in GRN 650 and GRN 735	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.	Supplement 1
Mutagenicity OECD 471	*S. typhimurium* (TA98, TA100, TA1535, and TA1537); *E. coli* WP2 *uvr*A	Up to 5000 ug/plate with & without metabolic activation	No significant differences in the mean number of revertant colonies in the presence or absence of metabolic activation between control and exposed groups.	Phipps *et al.* 2018	Under the test conditions, 2'-FL/DFL is not mutagenic.	Supplement 1
Mutagenicity OECD 471	*S. typhimurium* (TA1535, TA1537, TA98, and TA100); *E. coli* strain WP2 *uvrA*	Up to 5000 µg/plate with & without metabolic activation	No toxicity to any of the strains tested; no significant or dose related increase in revertant colonies; no mutagenic effect.	Van Berlo *et al.* 2018	Under the test conditions, 2'-FL is not mutagenic.	Supplement 1
Mutagenicity OECD 471	*S. typhimurium* (TA98, TA100, TA102, TA1535 and TA1537)	Up to 5000 µg/plate; with & without metabolic activation	No cytotoxicity; no increase in revertant colonies; no mutagenic effects.	Appendix M2 in GRN 000571	Under the test conditions, Jennewein 2'-FL is not mutagenic or cytotoxic.	GRN 000571
Mutagenicity OECD 474	Rat bone marrow cells	Oral; 500, 1000 or 2000 mg/kg bw 24 and 48 h post-administration	No signs of acute systemic toxicity; no mutagenic effects at any dose.	Appendix M1 in GRN 000571	Under the test conditions, Jennewein 2'-FL is not genotoxic and is not acutely toxic.	GRN 000571
Mutagenicity OECD 476	Mouse lymphoma cells (TK-locus)	up to 5000 µg/mL; with metabolic activation (4 h) & without (4 h, 8 h)	No cytotoxicity; no increase in mutant frequency.	Coulet *et al.* 2013	Under the test conditions, 2'-FL is not cytotoxic.	GRN 000571

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species/Sex/Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
Mutagenicity OECD 487	Peripheral human lymphocytes	Up to 2000 µg/mL with & without metabolic activation	No significant increase in the number of micronucleated cells in the presence or absence of metabolic activation.	Verbaan *et al.* 2015a as cited in GRN 650 and 735	Under the test conditions, 2'-FL is not mutagenic.	Supplement 1
Mutagenicity OECD 487	Peripheral human lymphocytes	Up to 2000 µg/mL with & without metabolic activation for 3 hours with a 27-hour harvest time or for 24 hours with a 24-hour harvest time	No significant increase in cytotoxicity or in the number of micronucleated cells in the presence or absence of metabolic activation.	Verbaan *et al.* 2015b as cited in GRN 650 and 735	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.	Supplement 1
Mutagenicity OECD 487	Human peripheral blood lymphocytes	Up to 2000 ug/mL (3 hours with and without metabolic activation or 20 hours without metabolic activation)	No biologically relevant differences in the percentage of micronucleated cells between control and exposed groups. No evidence of clastogenicity or aneugenicity.	Phipps *et al.* 2018	Under the test conditions, 2'-FL/DFL is not mutagenic.	Supplement 1
Mutagenicity OECD 487	Cultured binucleated human lymphocytes	Up to 2000 µg/mL with & without metabolic activation (4 hours treatment/20 hours recovery or 20 hours treatment and no recovery)	No cytotoxicity observed at any concentrations tested with or without metabolic activation; no significant dose-dependent increase in the number of binucleated cells containing micronuclei; no mutagenic effect	Van Berlo *et al.* 2018	Under the test conditions, 2'-FL is not mutagenic or cytotoxic.	Supplement 1
OECD 487	Cultured human peripheral lymphocytes	Up to 5000 µg/mL medium; with & without metabolic activation	No genotoxicity; no indications of chromosomal damage.	Appendix D	Under the test conditions, Jennewein 2'-FL is not genotoxic.	No
Repeated Dose Toxicity Studies						
90-day Oral Toxicity OECD 408	Neonatal rats/mf/10 per sex per group	Oral gavage; 0, 1000, 3000, or 5000 mg 2'-FL/DFL (2'-fucosyllactose and difucosyllactose in an 8:1 ratio)/kg bw/day for 90 days with 28-day recovery	**Mortalities**: No mortalities were observed. **Clinical signs**: No exposure related clinical signs were observed. **Body weight**: No significant or treatment related changes were observed. **Organ weights:** Relative kidney and seminal vesicle weight were significantly increased in males in the low-dose group.	Phipps *et al.* 2018	Under the test conditions 2'-FL did not induce toxic effects after repeated ingestion by rats.	Supplement 1

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species/Sex/Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
			Thymus weight was significantly increased for all male treatment groups, but no dose-response observed. Relative pituitary weights were significantly increased in females in the high-dose group at the end of the recovery period. **Sexual maturation and development** No exposure-related differences in the age or body weight at which the males and females attained physical signs of sexual maturation. The mean age for balano-preputial skinfold separation was slightly higher in highest-exposed males compared with vehicle controls. No differences in age of attainment of reflexes, startle response test, and mean ulna growth **Hematology and Clinical chemistry**: No test item-related or dose-responsive changes in hematology or clinical chemistry. **Neurotoxicity**: Functional observational battery and motor activity assessment did not indicate any neurotoxicity. **Histopathology:** No treatment-related macroscopic or microscopic changes were reported. **Urinalysis:** No biologically relevant or test item-related differences. **NOAEL:** Highest level tested of 5000 mg/kg bw/day			
90-day Oral Toxicity OECD 408	Rat/mf/40 per sex	0, 3, 6, or 10% (w/w) added to feed for 13 weeks	**Mortalities**: No exposure related mortalities were observed. **Clinical signs**: No exposure related clinical signs were observed. **Body weight**: No significant or treatment related changes were observed.	Van Berlo *et al.* 2018	Under the test conditions 2'-FL did not induce toxic effects after repeated ingestion by rats.	Supplement 1

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species/Sex/Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
			Organ weights: Relative liver weight was significantly increased in males in the high-dose group. Absolute and relative filled and empty cecum weights significantly increased in the mid- and high-dose males and females **Hematology and Clinical chemistry**: No treatment related changes in hematology or clinical chemistry. **Neurotoxicity**: Functional observational battery and motor activity assessment did not indicate any neurotoxicity. **Histopathology:** No treatment-related macroscopic or microscopic changes were reported. **NOAEL:** Highest level tested of ≥7.25 g/kg body weight/day in males and ≥7.76 g/kg body weight/day in females.			
90-day Oral Toxicity OECD 408	Rat/NR/NR	Oral gavage; 0, 2000, 4000, or 5000 mg 2'-FL/kg bw/day & FOS at 5000 mg/kg bw/day; 90 to 91 days with 28-day recovery	**Mortalities:** No exposure related mortalities were observed. **Clinical Signs:** Liquid feces in mid- and high-dose groups and reference groups; soiled urogenital areas in mid- and high-dose groups; hypersalivation, abnormal foraging and/or pedaling in mid- and high dose group and reference group. **Body weights and organ weights**: No significant changes in body weight, body weight gain, or food consumption reported; no changes in organ weights. **Other:** No toxicological effects noted in tibia length, reflex and physical development, time to sexual maturation, learning capacity memory, motor activity, exploratory behavior, or general movement. **Hematology and clinical chemistry**: No significant hematological changes; decreased	Penard *et al*. 2015 as cited in GRN 650	Under the test conditions 2'-FL did not induce toxic effects after repeated ingestion by rats.	Supplement 1

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species/Sex/Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
			triglyceride concentrations in the mid- and high-dose males; decreased cholesterol concentrations in all treated males and mid- and high-dose females; individual urea concentrations increased in high-dose group. Changes were within historical control ranges and were not observed following the recovery period. **Urinalysis:** No treatment related changes. **Macroscopic and histopathological evaluation:** No treatment related effects reported. **NOAEL:** The highest dose tested of 5000 mg/kg/day.			
Oral Toxicity, Repeated Dose OECD 408	Rat / mf / 100	Oral – gavage; 0, 2000, 5000, 6000 mg/kg bw & FOS at 6000 mg/kg bw – 90 days	**Mortalities:** 1 m and 1 f of high dose 2'-FL on day 2. 2 m of FOS group on days 12 & 13; 1 f of FOS group on day 108. Could not demonstrate relationship to treatment. **Clinical signs:** Diarrhea in all high-, mid-dose 2'-FL, & FOS animals, & several low-dose 2'-FL. Erythema in urogenital area high dose & FOS groups. Hyper-salivation in most high-dose 2'-FL & FOS groups, half of mid-dose 2'-FL group. **Body weight:** Transient lower weights in high- dose and FOS groups, not significant by study end. **Histopathology:** Higher incidence of minimal cortical tubular epithelial cytoplasmic vacuolation in kidneys of the mid- & high-dose 2'-FL and FOS groups. Not dose-dependent, not associated with clinical pathology changes, degeneration.	Coulet *et al.* 2013	Under the test conditions, the test substance did not induce toxic effects after repeated ingestion by rats. The results are consistent with other indigestible carbohydrates.	GRN 000571

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species/Sex/Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
			Findings considered unrelated to the 2'-FL by the authors.			
Oral Toxicity Pilot Study	Rat / f / 10	Oral – dietary; 10% in feed, ad libitum; 7 days	No mortalities; no change in behavior or appearance; no difference in food consumption or body weight from controls.	Appendix M3 in GRN 000571	Under the test conditions, Jennewein 2'-FL did not induce toxic effects after ingestion by female rats.	GRN 000571
Oral Toxicity, Repeated Dose OECD 408	Rat / mf / 64	Oral – dietary; 10% in feed, ad libitum (Mean = 7700 mg/kg bw in m; 8700 mg/kg bw in f); 90 days	No mortalities; no change in behavior or appearance; pale feces observed in approximately half of 2'-FL animals; no difference in food consumption or body weight from controls.	Appendix M4 in GRN 000571	Under the test conditions, Jennewein 2'-FL did not induce toxic effects after repeated ingestion by rats.	GRN 000571
Oral Toxicity, Pre- clinical	Pigs, neonatal farm / mf / 48	Oral – dietary; 0, 200, 500, and 2000 mg/L; 21 days	**Mortalities**: None. **Clinical signs:** No treatment related effects. **Body weight:** No treatment related effects. **Necropsy:** Microscopic findings: 1 m and 1 f in high-dose group, 1 f in mid-dose group exhibited mild to moderate inflammation within the keratinized portion of the squamous epithelium of the non-glandular part of the stomach. Another m of high-dose group exhibits focal loss/thinning of this area, but no ulceration. The authors considered these effects incidental and typical.	Hanlon & Thorsrud 2014; Appendix M5 in GRN 000571	Under the test conditions, Jennewein 2'-FL did not induce toxic effects after repeated ingestion by piglets.	GRN 000571
Clinical studies in infants						
Randomized, controlled, double-blind multicenter study	Infants age 14 ± 5 days; 30 infants in study population; 33 infants in control population	Ingestion of formula containing 0 or 0.25 g/L 2'-FL; 42 days	Outcomes based on questionnaires completed by caregivers **Primary Outcome** **Infant Gastrointestinal Symptom Questionnaire (IGSQ) score:** No significant	Storm *et al.* 2019	Formula containing 2'-FL was tolerated well based on a comprehensive tolerance assessment tool.	Supplement 1

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species/Sex/Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
			differences reported in IGSQ scores at baseline or at visit 1 at 42 weeks exposure. **Secondary Outcomes** **Stool frequency, consistency, and ease of passing:** No significant differences in stool frequency or consistency between the control and test group. Significantly more stools reported difficult to pass in the control groups compared to the test group. However, the number of infants with stool difficult to pass did not differ significantly between groups. **Spit up, Vomiting, Crying, and Fussing:** No differences in the occurrences of crying and fussing and vomiting frequency between groups. Proportion of infants to have any spit up did not differ between groups; however, in the infants reported to spit up, significantly more were reported to spit up > 5 times per day in the test groups compared to the control. **Formula intake**: Average intake of formula did not differ significantly between groups. **Adverse Events:** No serious adverse events reported. Spit up as an adverse event occurred in more test subjects compared to controls. Significantly more infections and infestations reported in the control group than in the treated groups.			
Multi-center, randomized, double-blind trial of two parallel groups	175 healthy full-term infants; 0 to 14 days old	Ingestion of formula containing 0 or 1 to 1.2 g/L 2'-FL for 6 months	No significant differences in weight gain between the test and control groups; mean weight, length, head circumference and body mass index (BMI) for all infants through age 4 months were comparable with the WHO standard growth curves; no changes in stool endpoints or composition of the microbiota in infants in the test group compared to controls; significantly lower incidences of bronchitis and	Puccio *et al*. (2017)	Formula containing 2'-FL was tolerated well	Supplement 1

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species/Sex/Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
			antibiotic use in treated infants compared to infants in the control group.			
Prospective, randomized, multi-center, double-blinded, controlled tolerance trial	131 Healthy term infants	Infant formula containing 0 (n = 30) or 0.2 g/L 2'-FL and 2 g/L short-chain fructooligosaccharide (scFOS) (n = 35) or human breast milk (n = 36); 35 days	No significant differences in stool consistency, formula intake, anthropometric measures, or percent feedings with spit-up/vomit associated with feeding among the three groups at 35 days of age; breast milk fed infants had a greater number of stools/day than formula fed infants.	Kajzer *et al.* (2016) as cited in Reverri *et al.* (2018)	No significant differences in gastrointestinal tolerance between infants fed formula containing 2'-FL and infants fed human breast milk were reported.	Supplement 1
Sub-study nested within the Marriage *et al.* (2015) study; and included the same study groups.	Healthy full-term singleton infants were enrolled by five days of age; 315 of the 424 originally enrolled in Marriage *et al*. (2015); 155 infants completed the study (39 Control formula, 37 Experimental formula 1, 37 Experimental formula 2, and 42 Human milk)	Ingestion of formula containing 0, 0.2, 1.0 g/L 2'-FL or human breast milk for 119 days	Infants fed formula supplemented with 2'-FL exhibited lower plasma and *ex vivo* inflammatory cytokine profiles, similar to those of a breastfed reference group. These findings indicate that 2'-FL supports aspects of immune development and regulation similar to that in a breastfed reference group.	Goehring *et al.* (2016)	Formula containing 2'-FL was tolerated well.	Supplement 1
Randomised, double-blind and controlled study.	Healthy full-term singleton infants were enrolled by five days of age; 424 originally enrolled; 304 infants completed the study (79 Control formula, 70 Experimental formula 1, 72 Experimental formula 2, and 83 Human milk)	Ingestion of formula containing 0, 0.2, 1.0 g/L 2'-FL or human breast milk for 119 days	No significant differences reported among any groups for weight, length or head circumference; 2'-FL was present in the plasma and urine of infants fed 2'-FL, and there were no significant differences in 2'-FL uptakes relative to the concentration fed; growth and 2'-FL uptakes were similar to those of breast-fed infants.	Marriage *et al.* (2015)	Formula containing 2'-FL was tolerated well.	Supplement 1

Table E1. Tabular Summary of Safety Studies reviewed in GRN 000571 and Supplement 1

Study Type	Species/Sex/Number	Route of Exposure; Dose - Duration	Observed Effects	Reference	Conclusionary Remarks	Previously Reviewed
Clinical studies in adults						
Double-blind, parallel, randomized, placebo-controlled study	100 health adults age 19 to 57 years (51 males and 49 females).	Diets supplemented with 5, 10 or 20 g of either 2'-FL, LNnT or 2'-FL+LNnT (2:1 mass ratio) or 2 g of glucose as the placebo each day at breakfast	44 participants reported a total of 56 mild adverse events usually a combination of symptoms including flatulence, bloating, and constipation. The most adverse events were reported by participants receiving the highest doses of 2'-FL and LNnT with flatulence being the most commonly reported adverse event followed by stomach pain, diarrhea or loose stool, and "rumbling"; reports of bloating and gas were significantly higher in the 20 g 2'-FL and LNnT groups; mean GSRS scores were low and participants receiving the highest dosages did not have statistically significant changes in their GSRS; participants receiving 20 g 2'-FL reported softer stools as compared to baseline; all measured clinical chemistry and hematology parameters remained within normal ranges.	Elison *et al.* (2016)	No adverse health effects were noted in adults consuming diets supplemented with up to 20 g 2'-FL per day.	Supplement 1

APPENDIX F
REFERENCES