SCG
Spherix Consulting Group

June 4, 2021

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

Dear GRAS Filing Team:

Enclosed is a GRAS Determination entitled "GRAS Determination for the Use of 2'-Fucosyllactose is Selected Conventional Foods and Enteral Tube Feeding Products". We believe that this GRAS Determination should be considered as a new notification because Chr. Hansen A/S intends to expand the use of its 2-fucosyllactose ingredient to selected conventional foods and enteral tube feeding formulas.

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

Sincerely,

[redacted]

Dietrich B. Conze, Ph.D.
Managing Partner

Enclosure: CD containing
- Form 3667
- Cover Letter
- GRAS Determination for the Use of 2'-Fucosyllactose in Selected Conventional Foods and Enteral Tube Feeding Formulas
- References

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GRAS Determination for the Use of 2'-Fucosyllactose in Selected Conventional Foods and Enteral Tube Feeding Formulas

Prepared for:

Chr. Hansen A/S[1]
9015 W Maple St.
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Prepared by:

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751 Rockville Pike, Unit 30-B
Rockville, MD 20852
USA

May 18, 2021

[1] Jennewein Biotechnology GmbH is now Chr. Hansen HMO GmbH. The legal entity (including the same company identification number), manufacturing premises, manufacturing processes and quality systems and certifications remains the same.

All documentation bearing the name of Jennewein Biotechnologie GmbH is in the process of being updated to Chr. Hansen HMO GmbH/Chr. Hansen A/S as appropriate. This is however an ongoing process; Chr. Hansen assures that the documents released with the Jennewein Biotechnologie GmbH's name, remain valid until the full update is completed.

Likewise, updated certificates and commercial registrations will be issued by the relevant competent authorities in due course; meanwhile the current certificates and commercial registrations remain valid until further notice.

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LIST OF ABBREVIATIONS

2'-FL: 2'-Fucosyllactose

3-FL: 3-Fucosyllactose

3'-SL: 3'-Sialyllactose

6'-SL: 6'-Sialyllactose

Alb: Albumin

ALT: Alanine aminotransferase

araA: Arabinose isomerase

BMI: Body mass index

BW: Body weight

CBPI: Cytokinesis-block proliferation index

CFR: United States Code of Federal Regulations

CFU: Colony forming units

CHO: Chinese hamster ovary cells

CI: Confidence interval

COSY: Correlation spectroscopy

DSMZ: Deutsche Sammlung für Mikroorganismen und Zellkulturen

DW: Dry weight

EDI: Estimated daily intake

EFSA: European Food Safety Authority

EU: Endotoxin unit

F6PPK: Fructose-6-phosphate phosphoketolase

FCC: Food Chemicals Codex

FDA: United States Food and Drug Administration

FFDCA: Federal Food, Drug, and Cosmetic Act

FOIA: Freedom of information Act

FOS: Fructooligosaccharides

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

Fru-6-P: Fructose-6-phosphate

FSSC: Food Safety System Certification

FUT: Fucosyltransferase

GI: Gastrointestinal

Glc-1-P: Glucose-1-phosphate

Glc-6-P: Glucose-6-phosphate

Gln-1-P: Glucosamine-1-phosphate

Gln-6-P: Glucosamine-6-phosphate

Glob: Gobulin

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

GMO: Genetically modified organism

GMP: Good manufacturing practices

GOS: Galactooligosaccharides

GRAS: Generally Recognized As Safe

GRN: GRAS Notification

HCD: Historical control data

HDL-C: High-density lipoprotein cholesterol

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

HMO: Human milk oligosaccharides

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

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

ICP-MS: Inductively coupled plasma mass spectrometry

IFNγ: Interferon gamma

LC-MS: Liquid chromatography coupled with mass spectrometry

LDL-C: Low-density lipoprotein cholesterol

LDPE: Low-density polyethylene

LNDFHI: Lacto-*N*-difucohexaose I

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

LNT: Lacto-N-tetraose

LOD: Limit of detection

LOQ: Limit of quantitation

MCH: Mean corpuscular hemoglobin

MCV: Mean corpuscular volume

ND: Not detected

NHANES: National Health and Nutrition Examination Surveys

NIH: National Institutes of Health

NMR: Nuclear magnetic resonance

NOAEL: No Observed Adverse Effect Level

OECD: Organization for Economic Cooperation and Development

PCR: Polymerase chain reaction

Ph Eur: European Pharmacopoeia

PHGG: Partially hydrolyzed guar gum

pLNnH: para-lacto-*N*-neohexaose

qPCR: Quantitative polymerase chain reaction

RI: Replicative index

TP: Total protein

UDP-Gal: UDP-galactose

UDP-Glc: UDP-glucose

UDP-GlcNAc: UDP-*N*-acetylglucosamine

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

A. SUBMISSION OF GRAS NOTICE

Chr. Hansen A/S is hereby submitting a GRAS notice in accordance with subpart E of part 170.

B. NAME AND ADDRESS OF THE SPONSOR

Chr. Hansen A/S
9015 W Maple St.
West Allis, WI 53214

C. COMMON OR USUAL NAME

2'-Fucosyllactose (2'-FL)

D. TRADE SECRET OR CONFIDENTIAL INFORMATION

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

E. INTENDED USE

Chr. Hansen A/S intends to use 2'-FL as an ingredient in toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas (Table 1).

Table 1. Comparison of Uses and Use Levels that are GRAS with the Intended Uses and Use Levels

Intended Uses	Intended Use Levels (g/kg or g/L)
Toddler formula (Go and Grow by Similac®)	2.4
Milk-based meal replacement beverages for children (Pediasure®)	12
Cereals, prepared, ready-to-serve, for infants and young children	12
Cereals, dry instant, for infants and young children	12
Bars, including snack bars, meal-replacement bars, and breakfast bars	12
Non-carbonated drinks (e.g. fitness water, thirst quenchers, sports and isotonic drinks)	6
Oral Electrolyte Solutions	1.2
Meal replacement drinks for adults including dairy and non-dairy drinks for weight reduction and formulas for pregnant women	12
Enteral tube feeds used as sole source nutrition (Ensure®, Glucerna®, and Boost®)	20

F. BASIS FOR GRAS DETERMINATION

This GRAS Determination for the use of 2'-FL for the intended use and use level specified above has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). The safety of the intake of 2'-FL has been determined to be GRAS by demonstrating that the safety of the intended level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food and is based on generally available and accepted information.

The use of 2'-FL as an ingredient for the intended use in selected toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. The subject of this GRAS Determination is a spray-dried, powdered food ingredient that contains not less than 90 % 2'-FL dry weight. The remaining components include carbohydrate by-products, ash, and moisture

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

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

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

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

    a. The subject of this GRAS Determination is manufactured using a genetically engineered strain of *Escherichia coli* BL21(DE3) by Chr. Hansen A/S in Food Safety System Certification (FSSC) 22000-, ISO 9001:2015-, GMP-, and International Featured Standards Food 6.1-compliant facilities. Chr. Hansen A/S is a Food Facility registered with FDA.

b. The genetically engineered strain of *Escherichia coli* BL21(DE3) used by Chr. Hansen A/S is non-toxigenic, not capable of DNA transfer to other organisms, and has the same virulence profile as *E. coli* BL21(DE3).

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

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

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

f. Use of the subject of this GRAS determination in the intended selected conventional foods and enteral tube feeding formulas results in mean and 90th percentile estimated daily intakes (EDIs) of 2.16 and 5.26 g/day (0.032 and 0.078 g/kg bw/day) for consumers not less than 2 years old.

g. Use of the subject of this GRAS determination in selected conventional foods and enteral tube feeding formulas results in mean and 90th percentile cumulative estimated daily intakes (EDIs) of 2.5 and 5.16 g/day (0.037 and 0.077 g/kg bw/day) for consumers not less than 2 years old.

h. The use of the subject of this GRAS determination in oral electrolyte solutions results in an estimated daily intake of 1.2-2.4 g of 2'-FL (equivalent to 88.9-177.8 mg of 2'-FL/kg bw/day assuming a 13.5 kg toddler and 17.1-34.3 mg of 2'-FL/kg bw/day assuming a 70 kg adult). Because OESs are intended for short term use, intake of 2'-FL from OESs will not impact the cumulative 2'-FL intake resulting from the use of 2'-FL in select conventional foods and enteral tube feeding formulas.

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

4. The safety of exposure to Chr. Hansen A/S's 2'-FL ingredient at its intended use level is supported by:

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

b. Published tolerance studies in neonatal piglets showing that the ingestion of up to 3.92 g/L of the subject of this GRAS determination alone or in the presence of other HMOs was well-tolerated and supported normal growth in neonatal piglets;

c. Clinical data showing the ingestion of HMOs are well tolerated in infants up to 1 g/day and adults up to 20 g/day;

d. Clinical data showing that the use of other non-digestible carbohydrates in infants, adults, enteral tube feeding products and oral electrolyte solutions is well tolerated up to 63 g/day;

e. The GRAS status of the subject of this GRAS Determination for use in non-exempt term infant formula (GRN 571);

f. The GRAS status of other 2'-FL products for use in non-exempt term infant formula, selected conventional foods and enteral tube feeding formulas (GRN 546, 2014; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019; GRN 897, 2020).

Therefore, the use of 2'-FL is safe and GRAS at the proposed level of addition to the toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas. 2'-Fucosyllactose is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

G. PREMARKET APPROVAL

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

H. AVAILABILITY OF INFORMATION

The data and information that serve as the basis for this GRAS determination will be available for review and copying at reasonable times at the office of Dietrich Conze, PhD, Managing Partner, Spherix Consulting Group Inc., at 751 Rockville Pike, Unit 30-B, Rockville, MD 20852; Telephone: 240-367-6089; Email: dconze@spherixgroup.com; or be sent to FDA upon request.

I. FREEDOM OF INFORMATION ACT (FOIA)

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

J. INFORMATION INCLUDED IN THE GRAS NOTIFICATION

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

[redacted]

Signature of Authorized Representative of
Chr. Hansen A/S

24 May 2021
Date

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

A. COMMON OR USUAL NAME

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

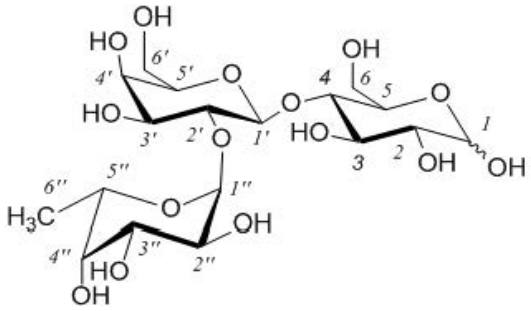
B. CHEMICAL NAME

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

C. MOLECULAR FORMULA AND MASS

$C_{18}H_{32}O_{15}$; 488.439 g/mol

D. STRUCTURAL FORMULA



E. DESCRIPTION OF 2'-FUCOSYLLACTOSE

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

F. PRODUCTION PROCESS

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

1. Description of the Production Strain

Because the subject of this GRAS Determination is the same as the subject of GRN 571 and the supplement of GRN 571, both which summarize the genetic engineering used to generate the current production organism *E. coli* BL21(DE3) #1242, also known as *JBT-2FLΔlacZ*, the descriptions of the genetic engineering provided in GRN 571 and the supplement to GRN 571 are incorporated by reference (see Appendix K, pg. 282-288 of GRN 571 and pg 3-6 of the supplement of GRN 571). Briefly, *JBT-2FLΔlacZ* was engineered from the early 2'-FL production strain #742, which lacks the genes encoding a β-galactosidase, an L-arabinose-isomerase, an L-fucose isomerase, an L-fuculokinase, an *N*-acetylglucosamine 6-phosphate deacetylase, a glucosamine 6-phosphate deaminase, a lipopolysaccharide biosynthesis protein, and a UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase and expresses the genes encoding a UDP-galactose-4-epimerase, a galactosyltransferase, a galactokinase, a galactose mutarotase, a sucrose hydrolase, a sucrose transporter, a fructokinase, a transcriptional regulator, a phosphomannomutase, a mannose-1-phosphate guanosyltransferase, a GDP-mannose-4,6-dehydratase, a GDP-L-fucose synthase, and a lactose permease. An α-1,2-fucosyltransferase and a heterologous 2'-FL exporter were then integrated into strain #742 to allow for synthesis and export of 2'-FL into the culture medium. The resulting integrants were subjected to two rounds of nitrosoguanidine (NTG) mutagenesis and screened for their ability to produce high levels of 2'-FL. The final production strain was designated as #1242 or *JBT-2FLΔlacZ* and has been deposited at DSMZ - German Collection of Microorganisms and Cell Cultures GmbH with the deposition number DSM 33609.

2. Manufacturing

a. Quality

Production of 2'-FL occurs in a contained, sterile environment at the Chr. Hansen A/S production facility in Maarweg 32, 53619 Rheinbreitbach, Germany, which is Food Safety System Certification (FSSC) 22000- and ISO 9001:2015-compliant, and an FDA-registered Food Facility (Registration # 1303109037512). Production also occurs at other Chr. Hansen A/S-

qualified manufacturers that are GMP-, ISO-, and International Featured Standards Food 6.1-compliant as shown by third-party audits.

b. Processing Aids and Food Contact Substances

All raw materials, processing aids, and food contact substances used to produce the 2'-FL powder are the same as those used to produce the 2'-FL that is the subject of GRN 571, which received a "no questions" letter from FDA, except that cobalt is no longer used in the culture medium. Therefore, the quality of the processing aids and raw materials and composition of the media described in GRN 571 (see pg. 17; Appendix E, pg. 99-144; Appendix J, pg. 280-281) are incorporated by reference. The water used throughout the manufacturing process complies with the TrinkwV, 2001 in Germany and the Council Directive 98/83/EC in the European Union and is non-fluoridated drinking water. The lactase used to degrade residual lactose in the fermentation medium complies with the recommended specifications of both the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) for food-grade enzymes (see pg. 21 of GRN 571 Supplement). All food contact surfaces (fermentation vessels and packaging materials) are either stainless steel or comply with the conditions of use that are specified in the US Code of Federal Regulations. The final powdered product is packaged in food-grade paper/low-density polyethylene (LDPE) bags in compliance with 21 CFR §177.1520. None of the processing aids are recycled or reused.

c. Production

The 2'-FL that is the subject of this GRAS Determination is manufactured using the same process described in GRN 571 and the GRN 571 supplement, except that cobalt is no longer added to the fermentation medium. Therefore, the detailed summary of the production process provided in GRN 571 (pg. 6-9) is incorporated by reference. Briefly, 2'-FL production involves three steps (Figure 1). During Step 1, *E. coli* BL21(DE3) *JBT-2FLΔlacZ* is cultured in minimal medium containing a carbon source (glucose, sucrose, or glycerol, or a combination thereof) and the substrate lactose, which is present throughout the process. Fermentation of lactose results in the production and secretion of 2'-FL into the culture medium. Step 2 involves purification and concentration of 2'-FL from the culture medium. Step 3 involves spray-drying of the 2'-FL concentrate, producing powdered 2'-FL. Additionally, lactase may be added at the end of the fermentation process to degrade excess lactose in the medium and increase product yield.

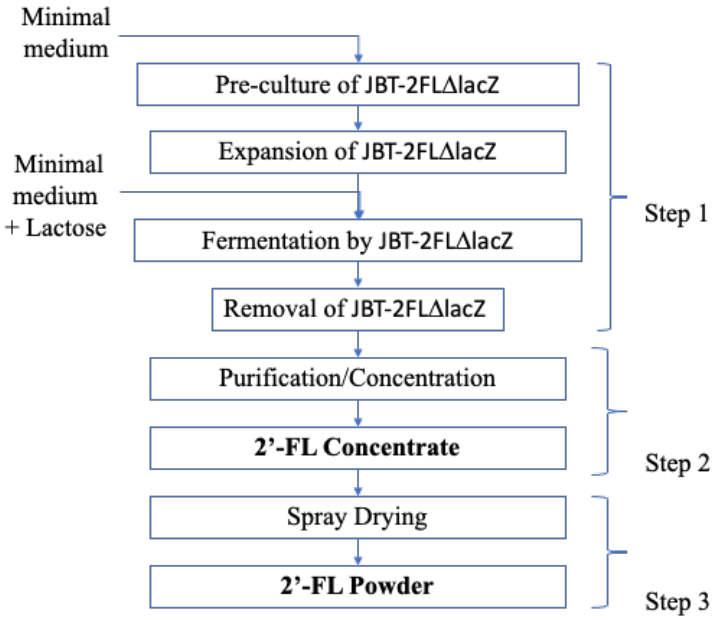


Figure 1. Production Process for 2'-Fucosyllactose.
JBT-2FLΔlacZ is expanded in minimal medium and with the addition of the lactose, 2'-fucosyllactose (2'-FL) is produced. The production strain/biomass is removed, yielding the oligosaccharide-containing fermentation medium. The medium is purified and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

G. FINISHED PRODUCT SPECIFICATIONS AND OTHER QUALITY ATTRIBUTES

To ensure a consistent food-grade product that is free of genetically-modified ingredients, each batch of powdered 2'-FL manufactured with the new production strain *JBT-2FLΔlacZ* is evaluated against the same product specifications that were established in GRN 571. The product specifications control the amount of 2'-FL, carbohydrate by-products, DNA and endotoxin residues derived from the production strain, heavy metals, and selected microbes. Each parameter is measured using the same compendial and/or internally validated, fit-for-purpose methods that were provided in GRN 571. Importantly, since the filing of GRN 571 and the GRN 571 supplement, Chr. Hansen A/S has learned that specifications for *Salmonella* serovars and *Cronobacter sakazakii* of absent in 25 g product and absent in 10 g of product, respectively, are sufficient to produce a safe food ingredients. Therefore, based on these observations, the specifications for *Salmonella* serovars and *Cronobacter sakazakii* have been changed to absent in 25 g product and absent in 10 g of product, respectively. Data from five batches of powdered 2'-FL show that the manufacturing process continues to reproducibly produce a product that meets the specifications that were established in GRN 571 (Table 2).

Table 2. Product Specifications and Batch Data for 2'-FL Powder

Parameter	Analytical method	Specification	Batch number				
			16130039	16116049	16151039	26108010	26120020
Physical Parameters							
Appearance (Color)[4]	Visual	White to ivory-colored	Complies	Complies	Complies	Complies	Complies
Appearance (Form)[4]		Spray-dried powder	Complies	Complies	Complies	Complies	Complies
Chemical Parameters							
2'-Fucosyllactose	HPAEC-PAD	≥ 90 % (%DW)	92.2	98.4	95.5	97.8	94.9
Lactose		≤ 5 % (% Area)	1.1	< 0.5	2.5	< 0.5	0.5
3-Fucosyllactose		≤ 5 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Difucosyllactose		≤ 5 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Fucosylgalactose		≤ 3 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	0.5
Glucose		≤ 3 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Galactose		≤ 3 % (% Area)	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Fucose		≤ 3 % (% Area)	0.7	< 0.5	1.8	< 0.5	0.7
Protein content[4]	Nanoquant (modified Bradford)	≤ 100 µg/g	< 10	< 10	< 10	< 10	< 10
Ash[1]	ASU L 06.00-4	≤ 0.5 %	< 0.01	0.03	0.08	< 0.01	0.08
Moisture[4]	KF titration	≤ 9.0 %	5.8	5.8	6.3	6.6	5.2
Endotoxins[3]	Ph. Eur. 2.6.14	≤ 300 EU/g	14	< 5	< 5	< 5	< 5
Aflatoxin M1[1]	DIN EN ISO 14501	≤ 0.025 µg/kg	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
GMO residues[2]	PCR	≤ 0.01%	Negative	Negative	Negative	Negative	Negative
Heavy Metals							
Arsenic[1]	ASU L 00.00-135 – ICP-MS	≤ 0.2 mg/kg	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Cadmium[1]		≤ 0.1 mg/kg	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010
Lead[1]		≤ 0.02 mg/kg	< 0.010	< 0.010	0.020	< 0.010	< 0.010
Mercury[1]		≤ 0.5 mg/kg	< 0.005	0.007	< 0.005	< 0.005	< 0.005
Microbiology							
Standard Plate Count[1]	ISO 4833-2	≤ 10000 cfu/g	< 10	< 10	30	20	< 10
Yeast and Mold[1]	ISO 21527-2	≤ 100 cfu/g	< 20	< 20	< 20	< 20	< 20
Coliform	ISO 4832	Absent/11 g	Absent	Absent	Absent	Absent	Absent
Enterobacteriaceae[1]	ISO 21528-1	Absent/11 g	Absent	Absent	Absent	Absent	Absent
Salmonella [1]	ISO 6579	Absent/25 g	Absent	Absent	Absent	Absent	Absent
Cronobacter sakazakii[1]	ISO/TS 22964	Absent/10g	Absent	Absent	Absent	Absent	Absent

Table 2. Product Specifications and Batch Data for 2'-FL Powder

Parameter	Analytical method	Specification	Batch number				
			16130039	16116049	16151039	26108010	26120020

Abbreviations: DW, dry weight; cfu, colony forming units; KF, Karl-Fischer; HPAEC-PAD, high performance anion exchange chromatography coupled with pulsed amperometric detection; PCR, polymerase chain reaction; ICP-MS, inductively coupled plasma mass spectrometry; EU, endotoxin unit; Ph Eur., European Pharmacopoeia; ND, not detected.

[1]Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory; ash limit of quantitation (LOQ) = 0.01 %. arsenic limit of detection (LOD) = 0.05 mg/kg; cadmium LOD = 0.01 mg/kg; mercury LOD = 0.005 mg/kg; lead LOD = 0.01 ppm; aflatoxin M1 LOQ = 0.025 µg/kg.

[2]Determined by GeneCon International GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory. Limit of detection = 0.01% of the finished product.

[3]Determined by Mikrobiologisches Labor. Dr. Michael Lohmeyer GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory; limit of quantitation = 5 EU/g.

[4]Determined by Chr. Hansen A/S using internally validated methods. Protein LOQ = 10 µg/g; carbohydrate by-products with a percent area greater than 0.5% (limit of quantitation) are considered.

H. STABILITY

1. Genetic Stability of the Production Strain

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

2. Stability of 2'-Fucosyllactose

As summarized in GRN 571, the subject of this GRAS Determination is stable for at least 104 weeks (2 years) when stored at 25 °C and 60% humidity, and for not less than 26 weeks (6 months) when stored at 40°C and 75% humidity in high density polyethylene (HDPE) bottles (see Section 2.4, pg. 27 -30). 2'-Fucosyllactose is also the subject of other GRAS Notifications and stability data provided in those GRAS Notification all support a 104-week shelf-life when stored at 25 °C and 60% humidity (GRN 546, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018).

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

2'-Fucosyllactose remained relatively unchanged throughout the 52-week testing period. Moisture content increased from 5.7% to 7.8%; however, the parameter did not exceed the product specification of not more than 9% at week 52 (Table 3).

Under accelerated conditions, 2'-FL decreased, and moisture increased over the course of the study, with moisture falling out of specification by 26 weeks (Table 4).

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

Table 3. Stability of 2'-Fucosyllactose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Ambient Conditions (25°C, 60% Relative Humidity)

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

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

Table 4. Stability of 2'-Fucosyllactose as a Component of a Mixed Human Milk Oligosaccharide Powder Under Accelerated Conditions (40°C, 75% Relative Humidity)

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

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

3. Stability of 2'-Fucosyllactose in Oral Electrolyte Solutions

As summarized in Table 5, initial data using 2'-FL at 200 mg/L show stability in OES for up to 14 months (Patent 10,695,358, date issued June 30, 2020, Abbott Laboratories).

Table 5. Stability of 2'-FL in Oral Electrolyte Solution*

Assay	Sample	Baseline	3 months	6 months	14 months
2'FL (mg/L)	Sterile 1	194.8	196.6	197.7	201.2
	Sterile 2	196.7	196.8	200.3	200.8
	Average	196.0	197.0	199.0	201.0

*Patent 10,695,358, date issued June 30, 2020 Abbott Laboratories; the target fortification for 2'-FL is 200 mg/L.

III. DIETARY EXPOSURE

A. INTENDED EFFECT

2'-Fucosyllactose is a non-digestible carbohydrate. The intended effect of adding 2'-FL to toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas is to increase 2'-FL intake.

B. HISTORY OF EXPOSURE

2'-Fucosyllactose is one of the most abundant oligosaccharides in human milk. It is also found in the milk of goats, pigs, chimpanzees, bonobos, and orangutans, and synthetic forms are used in infant formula, selected conventional foods, and enteral tube feeding formulas (Castanys-Muñoz et al., 2013; Chaturvedi et al., 2001).

In human milk, 2'-FL levels generally range from 0 to 9.5 g/L and vary with ethnicity, Secretor and Lewis-blood group status, lactation period, and term vs preterm birth (Table 6; Alderete et al., 2015; Austin et al., 2016; Austin et al., 2019; Azad et al., 2018; Chaturvedi et al., 1997; Kunz et al., 2017; Larsson et al., 2019; Leo et al., 2010; Ma et al., 2018; Marx et al., 2014; McGuire et al., 2017; McJarrow et al., 2019; Nijman et al., 2018; Paganini et al., 2019; Samuel et al., 2019; Sjögren et al., 2007; Williams et al., 2017; Spevacek et al., 2015; Sprenger et al., 2017; Thurl et al., 2010; Coppa et al., 2011; Gabrielli et al., 2011; Coppa et al., 2011; Erney et al., 2000; Nakhla et al., 1999; Asakuma et al., 2008; Chaturvedi et al., 2001; Smilowitz et al., 2013; Van Niekerk et al., 2014; Goehring et al., 2014; Hong et al., 2014). Some of these studies have also been evaluated in a systematic review conducted by Thurl et al. (2017), who calculated a mean concentration and 95% confidence limit of 2.74 g/L and 2.43 - 3.04 g/L, respectively, in the milk of secretor mothers.

In cow's milk, which is the most common milk used in the production of infant formula in the United States, the oligosaccharide content ranges from 100 to 1000 times lower than human milk and fucosylated oligosaccharides constitute less than 1% of the oligosaccharide fraction (Aldredge et al., 2013).

Synthetic forms of 2'-FL also exist, including the subject of this GRAS Determination, which are used in the infant formulas up to 2.4 g/L, selected conventional foods up to 600 g/kg, and enteral tube feeding formulas up to 6 g/L (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 815, 2019; GRN 852, 2019; GRN 897, 2020).

Thus, humans are exposed to 2'-FL either through the ingestion of human milk, cow's milk, and/or products containing synthetic forms of 2'-FL.

Table 6. Studies Determining the Concentration of 2'-Fucosyllactose (2'-FL) in Human Breast Milk

Study	Location	Number of Subjects/Samples	Lactation Timepoint(s)	2'-FL concentration
Alderete et al., 2015	United States	37 donors	1 and 6 months	Highest median ± interquartile range: 2.7 ± 3.7 g/L (1 month) Lowest median ± interquartile range: 2.4 ± 2.8 g/L (6 month) *Only median ± interquartile ranges were reported
Austin et al., 2016	China	450 donors (approximately 90 donors/timepoint)	Days 5-11, Days 12-30, Months 1-2, Months 2-4, Months 4-8	Reported range: 0.026 – 4.9 g/L Highest mean: 2.1 ± 1.4 g/L (days 5-11) Highest median: 2.1 g/L (days 5-11) Lowest mean: 1.1 ± 0.7 g/L (4-8 months) Lowest median: 1.2 g/L (4-8 months)
Austin et al., 2019	Switzerland	27 donors with 33 preterm infants (approx. 25 samples/timepoint) 34 donors with 34 term infants (approx. 28 samples/timepoint)	Weekly for 8 weeks after delivery (preterm and term) then every 2 weeks until 16 weeks (preterm only)	Reported range: 0.07 – 6.1 g/L Highest mean: 3.2 ± 1.9 g/L (Term, week 1) Highest median: 3.4 g/L (Term, week 1) Lowest mean: 1.3 ± 1.0 g/L (Preterm, weeks 12, 14 and 16) Lowest median: 1.3 g/L (Preterm, week 14)
Azad et al., 2018	Canada	427 donors	3- 4 months postpartum	Reported range: 0 – 6.76 g/L Mean: 2.2 ± 1.84 g/L Median: 2.4 /L
Berger et al., 2020	United States	50 donors	1 and 6 months postpartum	Reported range: 0 – 6.0 g/L
Chaturvedi et al., 1997	Mexico	50 donors	1-2 months postpartum	Mean: 1.7 ± 0.08 g/L *Only mean ± standard error was reported
Kunz et al., 2017	Spain	32 donors (21 secretors; 11 nonsecretors	Lactation days 1-7 (colostrum), 8-15 (transitional milk), and 16-30 (mature milk)	Highest median: 2.9 g/L (0 – 4.6 g/L) (Preterm, colostrum) Lowest median: 2.0 g/L (0 – 3.3 g/L) (Preterm, mature milk) *Only median and interquartile ranges were reported

Table 6. Studies Determining the Concentration of 2'-Fucosyllactose (2'-FL) in Human Breast Milk

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

Table 6. Studies Determining the Concentration of 2'-Fucosyllactose (2'-FL) in Human Breast Milk

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

Table 6. Studies Determining the Concentration of 2'-Fucosyllactose (2'-FL) in Human Breast Milk

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

C. INTENDED USES

2'-Fucosyllactose is GRAS for use in non-exempt, cow's milk-based term infant and toddler formulas up to 2.4 g/L; infant and toddler foods and toddler drinks up to 12 g/kg (solids) or g/L (liquids); baked goods and baking mixes; non-alcoholic beverages and beverage bases, breakfast cereals, jams and jellies, gelatins, puddings and fillings milk and milk products, dairy product analogs, grain products and pastas, processed vegetables, vegetable juices, processed fruits, fruit juices, bars, and enteral tube feeding formulas at use levels up to 600 g/kg (Table 7). The subject of this GRAS Determination is GRAS for use in term, cow's milk-based non-exempt infant formula at 2.0 g/L (GRN 571, 2015). Chr. Hansen A/S intends to expand the use of the subject of this GRAS Determination to toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas at levels ranging from 1.2 to 20 g/L (Table 7). Importantly, these expanded uses include new uses, substitutional uses for other forms of 2'-FL that are GRAS for use in infant formulas and conventional foods, and increases to 2'-FL use levels in uses that have already been determined GRAS. Therefore, a cumulative estimated daily intake must be calculated using the maximum use level for all uses to determine if Chr. Hansen A/S's expanded intended uses increase overall 2'-FL exposure.

Table 7. Comparison of Uses and Use levels That Are GRAS with the Intended Uses and Use Levels

Uses That Are GRAS[1]	Use Levels That are GRAS (g/kg or g/L)[1]	Intended Uses	Intended Use Levels (g/kg or g/L)	Maximum Use Level Used for Cumulative EDI Calculations (g/kg or g/L)
Non-exempt term infant formula	2.4	-	-	2.4
Toddler formula	2.4	Toddler formula (Go and Grow by Similac®)	2.4	2.4
Milk-based meal replacement beverages for children	2.4	Milk-based meal replacement beverages for children (Pediasure®)	12	12
Baby food, cereal	12	Cereals, prepared, ready-to-serve, for infants and young children	12	12
		Cereals, dry instant, for infants and young children	12	12
Baby food, yogurt	10	-	-	10
Baby snacks	57	-	-	57
Drinks for kids	1.2	-	-	1.2

Table 7. Comparison of Uses and Use levels That Are GRAS with the Intended Uses and Use Levels

Uses That Are GRAS[1]	Use Levels That are GRAS (g/kg or g/L)[1]	Intended Uses	Intended Use Levels (g/kg or g/L)	Maximum Use Level Used for Cumulative EDI Calculations (g/kg or g/L)
Breads and Baked Goods, gluten free	48	-	-	48
Ready-to-eat cereals, puffed	80	-	-	80
Ready-to-eat cereals, high-fiber	40	-	-	40
Ready-to-eat cereals, biscuit-type	40	-	-	40
Cereal, hot	31	-	-	31
Bars, snack	30	Bars, including snack bars, meal-replacement bars, and breakfast bars	12	30
Meal Replacement bars for Weight Loss	40			40
Carbonated Beverages	1.2	-	-	1.2
Flavored and Enhanced Water	1.2	-	-	1.2
Sports, Isotonic, and Energy Drinks	1.2	Non-carbonated drinks (e.g. fitness water, thirst quenchers, sports and isotonic drinks)	6	6
Oral electrolyte solutions	1.2	Oral electrolyte solutions	1.2	-[2]
Coffee	10	-	-	10
Tea	10	-	-	10
Fruit flavored drinks	1.2	-	-	1.2
Fruit juices and nectars	1.2	-	-	1.2
Vegetable juices	1.2	-	-	1.2
Meal replacement drinks (including dairy and non-dairy drinks for weight reduction)	5	Meal replacement drinks for adults including dairy and non-dairy drinks for weight reduction and formulas for pregnant women	12	12
Beverage whiteners	600	-	-	600
Unflavored, pasteurized milk	12	-	-	12
Buttermilk	1.2	-	-	1.2
Flavored Milk	1.2	-	-	1.2
Imitation milks	1.2	-	-	1.2
Yogurt	1.2	-	-	1.2
Non-dairy yogurt	12	-	-	12
Frozen desserts	17	-	-	17
Dairy puddings, custards, and mousses	17	-	-	17
Fruit pie filling	14.1	-	-	14.1

Table 7. Comparison of Uses and Use levels That Are GRAS with the Intended Uses and Use Levels

Uses That Are GRAS[1]	Use Levels That are GRAS (g/kg or g/L)[1]	Intended Uses	Intended Use Levels (g/kg or g/L)	Maximum Use Level Used for Cumulative EDI Calculations (g/kg or g/L)
Fruit filling in snacks	30	-	-	30
Syrups	7	-	-	7
Jellies and jams	60	-	-	60
Table-top sweeteners	1.2	-	-	1.2
Enteral and tube feeding formula	6	Enteral tube feeds used as sole source nutrition (Ensure®, Glucerna®, and Boost®)	20	20

[1]Obtained from GRN 546, GRN 571, GRN 650, GRN 735, GRN 749, GRN 852, GRN 897.
[2]Not included in the cumulative estimated daily intake calculation because the products are intended for short-term use only.

D. ESTIMATED DAILY INTAKE

1. Estimated Daily Intake of 2'-FL from Oral Electrolyte Solutions

Oral electrolyte solutions (OESs), such as Pedialyte, are specially formulated to replenish fluids and minerals and recommended to be used under medical supervision to prevent dehydration caused by vomiting, diarrhea, exercise, travel, or heat exhaustion. Conditions of use state the ingestion of 1-2 L of OES, such as Pedialyte, may be needed per day to maintain proper hydration, however, a medical professional should be consulted if vomiting, fever, or diarrhea continues beyond 24 hr or if consumption needs are greater than 2 L per day. Due to its infrequent use and low number of users within the database (1 user), calculation of an EDI using the National Center for Health Statistics' (NCHS) 2015-2016 National Health and Nutrition Examination Surveys (NHANES) is not appropriate.

A conservative EDI can be calculated from the intended use of OES. Consumption of a maximum of 1-2 L of an OES per day at a use level of 1.2 g of 2'-FL/L would result in a daily intake of 1.2-2.4 g of 2'-FL (equivalent to 88.9-177.8 mg of 2'FL/kg bw/day, assuming a 13.5 kg toddler and 17.1-34.3 mg of 2'FL/kg bw/day, assuming a 70 kg adult). Because OESs are intended for short term use, intake of 2'-FL from OESs will not impact the cumulative 2'-FL intake resulting from the use of 2'-FL in select conventional foods and enteral tube feeding formulas.

2. Estimated Daily Intake of 2'-FL from Selected Conventional Foods and Enteral Tube Feeding Formula

Estimates for the intake of Chr. Hansen A/S's intended uses of 2'-FL were based on the food uses and Chr. Hansen A/S's use level in Table 7, in conjunction with food consumption data included in the National Center for Health Statistics' (NCHS) 2015-2016 National Health and Nutrition Examination Surveys (NHANES) (CDC, 2018; USDA, 2018). Nutritional beverages such as Boost, Ensure, and Glucerna were used as surrogates for enteral and tube-feeding formulas. A total of 110 food codes representative of each approved use were chosen from the Food and Nutrition Database for Dietary Studies (FNDDS) for the corresponding biennial NHANES survey. Calculations from NHANES for the mean and 90th percentile intakes were performed for Chr. Hansen A/S's representative food uses of 2'-FL.

To determine the impact of Chr. Hansen A/S's intended uses on the cumulative estimated daily intake of 2'-FL from all uses, a cumulative estimated daily intake was calculated using the maximum use level for all uses with the food consumption data included in the National Center for Health Statistics' (NCHS) 2015-2016 National Health and Nutrition Examination Surveys (NHANES) (Table 7; CDC, 2018; USDA, 2018). A total of 1275 food codes representative of each approved use were chosen from the Food and Nutrition Database for Dietary Studies (FNDDS) for the corresponding biennial NHANES survey. As described previously, nutritional beverages such as Boost, Ensure, and Glucerna were used as surrogates for enteral and tube-feeding formulas. Calculations from NHANES for the mean and 90th percentile intakes were performed for all representative food uses of 2'-FL.

3. Food Consumption Survey Data

a. Survey Description

The most recent NHANES data for the years 2015-2016 are available for public use. NHANES are conducted as a continuous, annual survey, and are released in 2-year cycles. In each cycle, approximately 10,000 people across the U.S. completed the health examination component of the survey. Any combination of consecutive years of data collection is a nationally representative sample of the U.S. population. It is well established that the length of a dietary survey affects the estimated consumption of individual users and that short-term surveys, such as the typical 1-day dietary survey, overestimate consumption over longer time periods (Hayes et al., 2014). Because two 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) are available from the NHANES 2015-2016 survey, these data were used to generate estimates for the current intake analysis.

The NHANES provide the most appropriate data for evaluating food-use and food-consumption patterns in the United States, containing 2 years of data on individuals selected via stratified multistage probability sample of a civilian non-institutionalized population of the U.S. NHANES survey data were collected from individuals and households via 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all 4 seasons of the year. Day 1 data were collected in-person in the Mobile Examination Center (MEC), and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the desired degree of statistical independence. The data were collected by first selecting Primary Sampling Units (PSUs), which were counties throughout the U.S. Small counties were combined to attain a minimum population size. These PSUs were segmented and households were chosen within each segment. One or more participants within a household were interviewed. Fifteen PSUs are visited each year. For example, in the 2009-2010 NHANES, there were 13,272 persons selected; of these 10,253 were considered respondents to the MEC examination and data collection. 9754 of the MEC respondents provided complete dietary intakes for Day 1 and of those providing the Day 1 data, 8,405 provided complete dietary intakes for Day 2. The release data do not necessarily include all the questions asked in a section. Data items may have been removed due to confidentiality, quality, or other considerations. For this reason, it is possible that a dataset does not completely match all the questions asked in a questionnaire section. Each data file has been edited to include only those sample persons eligible for that particular section or component, so the numbers vary.

In addition to collecting information on the types and quantities of foods being consumed, the NHANES surveys collect socioeconomic, physiological, and demographic information from individual participants in the survey, such as sex, age, height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population.

Sample weights are incorporated with NHANES surveys to compensate for the potential under-representation of intakes from specific population groups as a result of sample variability due to survey design, differential non-response rates, or other factors, such as deficiencies in the sampling frame (CDC, 2006; USDA, 2012).

b. Statistical Methods

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer in Octave and used to generate estimates for the intake of 2'-FL by the U.S. population. Estimates for the daily intake of 2'-FL represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES data; these

average amounts comprised the distribution from which mean and percentile intake estimates were produced. Mean and percentile estimates were generated incorporating sample weights to provide representative intakes for the entire U.S. population. "All-user" intake refers to the estimated intake of 2'-FL by those individuals consuming food products containing 2'-FL. Individuals were considered users if they consumed one or more food products containing 2'-FL on either Day 1 or Day 2 of the survey.

4. Food Usage

The estimated "all-user" total intakes of 2'-FL from Chr. Hansen A/S's intended uses only from 110 proposed food uses listed in NHANES in the U.S. by population group is described in Table 8. In summary, 9.67% of the total U.S. population 2+ years was identified as consumers of Chr. Hansen A/S's intended uses of 2'-FL in the 2015-2016 survey. The mean intakes by 2'-FL consumers age 2+ from Chr. Hansen A/S's intended food uses were estimated to be 2.16 g/person/day or 0.032 g/kg body weight/day. The heavy consumer (90th percentile) intakes were estimated to be 5.26 g/person/day or 0.078 g/kg body weight/day. The highest consumers on a mean EDI by body weight basis were ages 13 months to 2 years at 0.077 g/kg body weight/day (0.970 g/day).

The cumulative estimated "all-user" total intakes of 2'-FL from 1,275 proposed food uses listed in NHANES in the U.S. by population group is described in Table 9. In summary, 83.4% of the total U.S. population 2+ years was identified as consumers of 2'-FL from the selected food uses in the 2015-2016 survey. The mean intakes by all 2'-FL consumers age 2+ from all 2'-FL food uses were estimated to be 2.50 g/person/day or 0.037 g/kg body weight/day. The heavy consumer (90th percentile) intakes were estimated to be 5.16 g/person/day or 0.077 g/kg body weight/day. The highest consumers on a mean EDI by body weight basis were ages 13 months to 2 years at 0.108 g/kg body weight/day (1.35 g/day).

Importantly, a comparison of the mean and 90th percentile EDIs of 2'-FL ages 2+ from Chr. Hansen A/S's food uses and all food uses shows that the mean EDI increases slightly from 2.16 to 2.50 g/day and the 90th percentile EDI decreases from 5.26 to 5.16 g/day, which is likely due to the broad range of uses and an increase in the number of users (Table 7; compare Tables 8 and 9, respectively). Thus, Chr. Hansen A/S's intended uses and use levels do not significantly increase 2'-FL cumulative exposure.

Table 8. Estimated "All-user" Daily Intake (EDI) of 2'-FL from Chr. Hansen A/S's Food Uses by Population Group (2015-2016 NHANES Data)

Population Group	N users	N population	% Users	Mean mass (kg)	Mean EDI (g)	90th % EDI (g)	Mean EDI (g/kg)	90th % EDI (g/kg)
ages 0-6 months	49	197	24.88	7.00	0.104	0.182	0.015	0.026
ages 7-12 months	72	207	34.78	9.44	0.331	0.776	0.035	0.082
ages 13 months-2 years	44	535	8.22	12.56	0.970	2.190	0.077	0.174
ages 2-5 years	69	915	7.54	16.92	1.174	2.976	0.069	0.176
ages 6-12 years	146	1505	9.70	36.58	1.594	3.660	0.044	0.100
ages 13-19 years	145	1143	12.69	67.35	2.09	4.929	0.031	0.073
ages 20 years and up	513	5748	8.92	80.76	2.41	5.490	0.030	0.068
ages 2 years and up	873	9311	9.67	67.35	2.16	5.257	0.032	0.078

Table 9. Cumulative Estimated "All-user" Daily Intake (EDI) of 2'-FL in All Food Uses by Population Group (2015-2016 NHANES Data)

Population Group	N users	N population	% Users	Mean mass (kg)	Mean EDI (g)	90th % EDI (g)	Mean EDI (g/kg)	90th % EDI (g/kg)
ages 0-6 months	138	197	70.05	7.00	0.323	0.512	0.046	0.073
ages 7-12 months	171	207	82.61	9.44	0.622	1.54	0.066	0.163
ages 13 months-2 years	403	535	75.33	12.56	1.35	2.93	0.108	0.233
ages 2-5 years	641	915	70.06	16.92	1.26	2.93	0.074	0.173
ages 6-12 years	1140	1505	75.75	36.58	1.40	2.96	0.038	0.081
ages 13-19 years	975	1143	85.30	67.35	1.98	4.28	0.029	0.063
ages 20 years and up	4775	5748	83.07	80.76	2.90	5.93	0.036	0.073
ages 2 years and up	7531	9311	83.42	67.35	2.50	5.16	0.037	0.077

IV. SELF-LIMITING LEVELS OF USE

This part does not apply.

V. COMMON USE IN FOOD BEFORE 1958

This part does not apply.

VI. NARRATIVE ON THE CONCLUSION OF GRAS STATUS

The subject of this GRAS Determination is a synthetic form of 2'-FL, which is a non-digestible oligosaccharide found in human milk, also known as a human milk oligosaccharide (HMO). Published studies indicate that the levels of 2'-FL in human milk range from 0 to 9.5 g/L with means and medians ranging from 0.01 to 3.8 and 0.01 to 5.2 g/L, respectively.

To obtain a thorough and comprehensive understanding of the safety of 2'-FL per the intended uses and use levels, searches of the published scientific literature were conducted using Pubmed. All articles published up to May 10, 2021 that evaluated the safety of 2'-FL in conventional food, oral electrolytes solutions (OESs), and enteral tube feeding formulas were retrieved and reviewed. Consistent with the requirements of the GRAS standard, Chr. Hansen A/S considered the totality of publicly available data and information relevant to the safety of 2'-FL including the use of other HMOs in selected conventional foods and oral electrolyte solutions, and non-digestible carbohydrates in enteral tube feeding products. This document includes the entire results of these searches.

Currently, seven synthetic forms of 2'-FL are GRAS for use in non-exempt term infant formulas, selected conventional foods, and enteral tube feeding formulas (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019; GRN 897, 2020). The subject of this GRAS determination is the same as the subject of GRN 571 and a supplement to GRN 571 and is GRAS for use in non-exempt term infant formula. As summarized in the supplement to GRN 571, the safe use of 2'-FL in non-exempt term infant formula is supported by published and unpublished toxicological studies conducted with either the subject of this GRAS Determination or the subject of other 2'-FL GRAS Determinations, as well as corroborative neonatal piglet studies. 2'-Fucosyllactose is not genotoxic, has a no observed adverse effect level (NOAEL) of at least 5 g/kg/day, and is well tolerated in neonatal piglets. When administered in combination with other HMOs, 2'-FL has a NOAEL of 2.67 and 3.28 g/kg bw/day in male and female rats and is well-tolerated in neonatal piglets. Additionally, publicly available clinical data show that the ingestion of 2'-FL, other HMOs, such as 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL), and other non-digestible carbohydrates are also well tolerated in infants, children, and adults, oral electrolyte solutions, and susceptible population groups that receive enteral tube feeding formulas. Importantly, because infants are considered a susceptible population group from a safety perspective, the subject of this GRAS Determination is GRAS for use in non-exempt term infant formula, and other 2'-FL products have been determined safe for use in selected conventional foods and enteral tube feeding formulas (Scientific Committee on Food, 1998; GRN 546, 2014; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019; GRN 897, 2020), there is reasonable

certainty that the use of the subject of this GRAS Determination per the intended uses will also be safe in children, adults, and enteral tube feeding formulas. Chr. Hansen A/S therefore concludes that the subject of this GRAS Determination is GRAS as an ingredient in toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas at the intended use level.

A. SAFETY OF THE PRODUCTION ORGANISM

The safety of the host organism, *E. coli* BL21(DE3), is thoroughly summarized in GRN 571 (Appendix K, pg. 282-300), and the GRN 571 Supplement, all of which received "no questions" letters from the FDA. GRN 571 describe the use of *E. coli* BL21(DE3) as the host organism in the production of BbgIV beta-galactosidase and 2'-FL, respectively.

Escherichia coli are commensal residents of the gut microflora of humans and numerous animal species. *E. coli* strains are taxonomically grouped into 5 different phylogroups (A, B1, B2, D, and E) based on the sequence similarity of housekeeping genes (Archer et al., 2011). Human commensal strains are typically found in Group A or B1, with non-related pathogenic strains classified under Group B2, D, and E. Three group A laboratory strains as well as strains K-12, B, C, and their derivatives are designated as Risk Group 1 organisms according to their relative pathogenicity for healthy adult humans (Archer et al., 2011; Daegelen et al., 2009). Under current National Institutes for Health (NIH) guidelines for research involving recombinant or synthetic nucleic acid molecules, Risk Group 1 organisms "are not associated with disease in healthy adult humans" (National Institutes of Health, 2019). Of these strains, *E. coli* K-12 and the B derivatives (*e.g.*, BL21) are among the most widely used for production of industrial, pharmaceutical, and food biotechnology preparations.

Several comprehensive studies have demonstrated the safety of *E. coli* BL21(DE3). This strain does not carry the well-recognized pathogenic components required by *E. coli* strains that cause the majority of enteric infections. *E. coli* BL21(DE3) is therefore considered to be non-pathogenic and unlikely to survive in host tissues or to cause disease (Chart et al., 2000). *E. coli* BL21(DE3) was one of the first organisms to have its complete genome sequence assembled and differs only marginally from another widely used production strain, *E. coli* K-12 (Studier et al., 2009). This sequencing revealed the absence of genes encoding invasion factors, adhesion molecules, and enterotoxins associated with virulence (Jeong et al., 2009). Finally, an acute oral toxicity study showed that the *E. coli* BL21(DE3) endotoxin produced no toxicity in mice, even at the highest dose of 1,000,000 EU (3.3 mg/kg body weight) (Harper et al., 2011). Importantly, because *JBT-2FLΔlacZ* was engineered with genes with known function, which do not confer

toxicogenicity, virulence, or DNA, using site-specific homologous recombination or transposition, *JBT-2FLΔlacZ* is non-toxigenic, not capable of DNA transfer to other organisms, and has the same virulence profile as *E. coli* BL21(DE3).

Based on the comprehensive characterization of this strain and its widespread use in protein production, the use of *E. coli* BL21(DE3) as the host strain and *JBT-2FLΔlacZ* as the production strain are not expected to result in any safety concerns.

B. ABSORPTION, DISTRIBUTION, METABOLISM, AND EXCRETION

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

For 2'-FL specifically, two studies that evaluated its ADME have been published since the filing of GRN 571, Vazquez et al. (2017) and Marriage et al. (2015). Vazquez et al. (2017) evaluated the kinetics and metabolic fate of absorbed 2'-FL in rats. 2'-Fucosyllactose (0.2, 1.0, or 5 g/kg) was administered by gavage and lacto-*N*-neotetraose (LNnT), lactose, fucose, sialic acid, 2'-FL, 6'-SL, and 3'-SL levels were quantitated in serum and urine over the course of 300 minutes by ultra-performance liquid chromatography coupled mass spectrometry (LC-MS). 2'-Fucosyllactose was detected in the serum of 13% of the animals at baseline (0.045 +/- 0.2 μg 2'-FL/mL serum). Following gavage, serum 2'-FL levels increased to a maximum of approximately

7 μg/mL 60 minutes after the administration of 0.2 g 2'-FL/kg, 20 μg/mL 180 minutes after the administration of 1 g 2'-FL/kg, and 45 μg/ml 60 minutes after the administration of 5 g 2'-FL/kg. After the maximum concentrations were reached following the administration of 0.2 and 1 g 2'-FL/kg, the levels then declined, but did not reach baseline by 300 minutes. Once the maximum concentration was reached after the administration of 45 μg/mL, the serum levels remained constant over the following 240 minutes. Additionally, the levels of the parent sugars of 2'-FL, lactose and fucose increased in plasma after the administration of 2'-FL, particularly following the administration of 5 g/kg. Urinalysis revealed that the 2'-FL levels were low at baseline and increased dose-dependently between 90 and 120 min post gavage. Additionally, urinary levels of lactose and fucose also increased after the 2'-FL gavage. Together, these data confirm the results of previous studies that small amounts of 2'-FL are absorbed and metabolized at least in part to lactose either prior to and/or after absorption.

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

Three hundred thirty-eight infants completed the study, 304 of whom completed the study on the assigned feeding or human milk. The number of premature discontinuations on the study formulas was not different among the formula-fed groups. No 2'-FL was detected in the plasma of infants fed the standard milk-based commercial formula containing GOS, whereas 2'-FL was detected in the plasma and urine of infants provided the 2'-FL-supplemented formula and in infants consuming human milk, with the greatest mean 2'-FL plasma and urine concentrations in the infants fed human milk and the formula containing 1.0 g 2'-FL/L. Based on

these results, Marriage et al. concluded that the absorption of 2'-FL in infants fed 2'-FL-supplemented infant formulas is similar to that in breast-fed infants.

Importantly, because other 2'-FL products are GRAS for use in selected conventional foods and enteral formula and the subject of this GRAS Notification is structurally identical to the 2'-FL found in breast milk, there is reasonable certainty that the absorption, distribution, metabolism, and excretion of 2'-FL ingested from the intended uses at the intended use levels will mimic those from other sources of 2'-FL.

C. TOXICOLOGY

The safety of 2'-FL is supported by numerous unpublished and published genotoxicity, subchronic toxicity, neonatal piglet tolerance studies (Table 10; Coulet et al., 2014; Jennewein, 2013; Jennewein, 2014a; Jennewein, 2014b; Jennewein, 2014c; Verspeek-Rip, 2015; Verbaan, 2015a; Verbaan, 2015b; van Berlo et al., 2018; Phipps et al., 2018; Pernard, 2015; Parschat et al., 2020; Hanlon and Thorsrud, 2014; Hanlon, 2020). Four unpublished studies and one published 21-day neonatal piglet tolerance study were conducted to support the safety and GRAS status of the 2'-FL that is the subject of this GRAS Determination and were summarized in GRN 571 (Jennewein, 2014a; Jennewein, 2014b; Jennewein, 2013; Jennewein, 2014c; Hanlon and Thorsrud, 2014). Since the Agency's "no questions" letter to the GRN 571 supplement, which summarized the genotoxicity and subchronic toxicity studies published since the filing for GRN 571 (Verspeek-Rip, 2015; Verbaan, 2015; Verbaan, 2015; van Berlo et al., 2018; Phipps et al., 2018; Pernard, 2015), two new published studies conducted by Parschat et al., (2020) and Hanlon (2020) evaluated the genotoxicity, subchronic toxicity, and tolerance of a mixture of HMOs containing 2'-FL manufactured by Chr. Hansen A/S. Because the studies published since the filing of GRN 571 have been extensively summarized in previous GRAS Notifications and the supplement to GRN 571 (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 815, 2019), all of their summaries are incorporated by reference and briefly summarized in tabular format below along with the detailed summaries of the new studies conducted by Parschat et al. (2020) and Hanlon et al. (2020). Collectively, all of the studies show that 2'-FL alone or in the presence of other HMOs is not mutagenic, clastogenic or aneugenic, has a NOAEL of at least 5 g/kg/day in rats and is well-tolerated up to 1.6 g/kg in neonatal piglets.

Table 10. Toxicity Studies that Support the Use of 2'-Fucosyllactose in Infant Formula

Publication	Test Substance	Method of Manufacturing	Manufacturer	Study Type	Conclusions	GRAS Notice
Genotoxicity Studies						
Coulet et al. (2014)	2'-Fucosyllactose (2'-FL)	Chemical synthesis	Glycom	An OECD-compliant bacterial reverse mutation test	2'-FL is not mutagenic	546
				An OECD-compliant *in vitro* mammalian cell gene mutation assay in mouse lymphoma L5178Y cells	2'-FL is not mutagenic	546
Jennewein (2014b)(Unpublished)	2'-FL	Fermentation	Jennewein Biotechnology, GmBH	An OECD-compliant bacterial reverse mutation test	2'-FL is not mutagenic	571
Jennewein (2014a)(Unpublished)	2'-FL	Fermentation	Jennewein Biotechnology, GmBH	An OECD-compliant micronucleus test	2'-FL is not clastogenic or aneugenic	571
Verspeek-Rip (2015)(Unpublished)	2'-FL	Fermentation	Glycom	An OECD-compliant bacterial reverse mutation test	2'-FL is not mutagenic	650
Verbaan (2015a)(Unpublished)	2'-FL	Chemical synthesis	Glycom	An OECD-compliant *in vitro* micronucleus test in human peripheral lymphocytes	2'-FL is not clastogenic or aneugenic	650
Verbaan (2015b) (Unpublished)	2'-FL	Fermentation	Glycom	An OECD-compliant *in vitro* micronucleus test in human peripheral lymphocytes	2'-FL is not clastogenic or aneugenic	650
van Berlo et al. (2018)	2'-FL	Fermentation	Friesland Campina Domo	An OECD-compliant bacterial reverse mutation test	2'-FL is not mutagenic	735
				An OECD-compliant *in vitro* micronucleus test in cultured human lymphocytes	2'-FL is not clastogenic or aneugenic	735
Phipps et al. (2018)	2'-FL and difucosyllactose (DFL)	Fermentation	Glycom A/S	An OECD-compliant bacterial reverse mutation test	2'-FL/DLF is not mutagenic	815
				An OECD-compliant *in vitro* mammalian micronucleus test in human blood lymphocytes	2'-FL/DLF is not mutagenic	815
Parschat et al. (2020)	2'-FL, 3-fucosyllactose (3-FL), lacto-N-tetraose (LNT), 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL)	Fermentation	Jennewein Biotechnology, GmBH	An OECD-compliant bacterial reverse mutation test	2'-FL is not mutagenic	921
				An OECD-compliant chromosomal aberration test	2'-FL is clastogenic, or aneugenic	921

Table 10. Toxicity Studies that Support the Use of 2'-Fucosyllactose in Infant Formula

Publication	Test Substance	Method of Manufacturing	Manufacturer	Study Type	Conclusions	GRAS Notice
Subchronic Toxicity Studies						
Coulet et al. (2014)	2'-FL	Chemical synthesis	Glycom	A 14-day oral toxicity range finder study in rats An OECD-compliant 90-day oral toxicity study in rats	NOAEL: 5 g/kg/day	546
Jennewein (2013) (Unpublished)	2'-FL	Fermentation	Jennewein Biotechnology, GmBH	A 7-day dietary toxicity study in rats	NOAEL: males=7.6 g/kg/day; females=8.72 g/kg/ day	571
Jennewein (2014c)(Unpublished)				An OECD-compliant 90-day dietary toxicity study in rats		
(Pernard, 2015) (Unpublished)	2'-FL	Fermentation	Glycom	An OECD-compliant 90-day dietary toxicity study in rats	NOAEL: 5 g/kg/day	650
van Berlo et al. (2018)	2'-FL	Fermentation	Friesland Campina Domo	An OECD-compliant 90-day dietary toxicity study in rats	NOAEL: males=7.25 g/kg/ day; females=7.76 g/kg/ day	735
Phipps et al. (2018)	2'-FL and DFL	Fermentation	Glycom A/S	An OECD-compliant 90-day oral toxicity study in rats	NOAEL: 5 g/kg/day	815
Parschat et al. (2020)	2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL	Fermentation	Jennewein Biotechnology, GmBH	A 7-day dietary toxicity study in rats An OECD-compliant 90-day dietary toxicity study in rats	NOAEL: males = 5.67 g/kg bw/day; females = 6.97 g/kg bw/day	921
Neonatal Piglet Tolerance Studies						
Hanlon and Thorsrud (2014)	2'-FL	Fermentation	Jennewein Biotechnology, GmBH	21-day in neonatal piglet tolerance study	NOAEL: males = 0.29 g/kg/ day; females = 0.29 g/kg/ day	571
Hanlon (2020)	2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL	Fermentation	Jennewein Biotechnology, GmBH	21-day in neonatal piglet tolerance study	NOAEL: males = 1.6 g/kg/day; females = 1.7 g/kg/ day	921

1. Genotoxicity

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

i. Bacterial Reverse Mutation Tests

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

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

ii. Micronucleus Tests

As summarized in the GRN 571 supplement, van Berlo et al. (2018) evaluated the clastogenic and aneugenic effects of 2'-FL produced by Friesland Campina Domo using fermentation in an OECD 487-compliant *in vitro* micronucleus test using cultured human lymphocytes. Duplicate cultures of binucleated human lymphocytes, in the absence and presence of a metabolic activation system, were exposed to concentrations of 2'-FL ranging from 3.9 to 2000 µg/mL. Cytotoxicity was determined using the Cytokinesis-Block Proliferation Index. In the first experiment, exposure was for 4 hours with a 20-hour recovery time, and in the second experiment, exposure was for 20 hours with no recovery time. Results indicated no statistically significant, dose-related increases in cytotoxicity or in the number of binucleated cells containing micronuclei at any concentration tested in experiment 1 or 2. The number of binucleated cells containing micronuclei was reported to be within the test facility's historical data range. The authors conclude that 2'-FL is not mutagenic based on the negative results of the *in vitro* micronucleus test.

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

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

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

i. Bacterial Reverse Mutation Tests

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

To evaluate the mutagenicity of an HMO mixture containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Chr. Hansen A/S using fermentation, Parschat et al. (2020) conducted an OECD 471-compliant bacterial reverse mutation test. Five strains of *S. typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) were used in two independent experiments with and without metabolic activation. The first experiment was conducted as a plate incorporation test and the second as a preincubation test (Ames et al., 1973;

Ames et al, 1975; Maron and Ames, 1983). Five, 10.0, 31.6, 100, 316, or 600 mg of the mixture containing 2.4, 4.7, 14.9, 47.1, 148.8, and 282.6 mg 2'-FL, respectively, were applied to each plate. Purified water was the negative control and the positive controls for the different strains were sodium azide (for TA1535 and TA100), 2-nitrofluorene (for TA98), benozo[a]pyrene 9AA (for TA1537, and mitomycin C (for TA102). Cytotoxicity was defined as a reproducible reduction in the number of colonies by more than 50% compared to the solvent control and/or a scarce background lawn. Compared to the negative control, the positive controls increased the mean revertant colony numbers at least threefold with and without metabolic activation, verifying the validity of the test. For the HMO mixture, no cytotoxicity or mutagenicity was noted in any of the test strains up to 600 mg HMO mixture/plate (equivalent to 282.6 mg 2'-FL/plate) in either the plate incorporation or preincubation tests (Table 11). Parschat et al. concluded that the results indicate that the HMO mixture, and the 2'-FL contained therein, was not mutagenic under the conditions tested.

Table 11. Bacterial Reverse Mutation Test Performed with an HMO Mixture Containing 47.1% 2'-Fucosyllactose[c]

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

Abbreviations: BaP, benozo[a]pyrene; 2-AA, 2-aminoanthracene; 2-NF, 2-nitrofluorene; 9-AA, 9-aminoacridine; NaN_3, sodium azide.
Values are means (n=3) ± standards deviations.
[a] Positive controls without S9: NaN_3 for TA1535 and TA100, 2-NF for TA98, 9-AA for TA1537, mitomycin C for TA102.
[b] Positive controls with S9: BaP for TA98, TA102 and TA1537, 2-AA for TA100 and TA1535.
[c]The HMO mixture also contained 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Chr. Hansen A/S.

ii. Micronucleus tests

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

To evaluate the clastogenicity and/or aneugenicity of an HMO mixture containing 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Chr. Hansen A/S using fermentation, Parschat et al. (2020) performed an OECD 408-compliant *in vitro* micronucleus test using human peripheral blood lymphocytes. Peripheral blood lymphocytes were obtained by venipuncture from young, healthy, non-smoking individuals with no known recent exposures to genotoxic chemicals or radiation and exposed to 7.5, 15, 30, and 60 mg HMO mixture/mL medium (equivalent to 3.5, 7.1, 14.1, and 28.3 mg 2'-FL/mL medium) for 4 or 24 hrs in the presence and absence of metabolic activation. Purified water was the negative control and the positive controls were mitomycin C (0.2 μg/mL), colchicine (0.02 μg/mL), and cyclophosphamide (20 μg/mL) with and/or without metabolic activation. At least 500 cells per replicate cell culture were scored and classified as mononucleates, binucleates, or multinucleates to estimate the proliferation index as a measure of toxicity. The evaluation of cytotoxicity was based on the Cytokinesis-Block Proliferation Index (CBPI) or the Replicative Index (RI). The CBPI indicates the average number of nuclei per cell during the period of exposure to CytoB and is used to calculate cell proliferation. The RI indicates the relative number of cell cycles in treated cultures compared to control cultures and can be used to calculate the percentage of cytostasis. Micronucleus frequencies were analyzed in at least 2000 binucleate cells per concentration (*i.e.*, ≥ 1000 binucleate cells per culture; two cultures per concentration). The ability of the HMO mix to induce micronuclei was considered to be positive if there was a statistically significant and/or dose related increase compared to the negative control or if any of the results were outside the distribution of the historical negative control data (Poisson-based 95% control limits). Mitomycin C and cyclophosphamide induced significant chromosomal damage whereas colchicine induced significant ($p \leq 0.05$) damage to the cell division apparatus (Table 12), both validating the tests. In contrast, no chromosomal damage was observed with the HMO mixture at any concentration or under any condition tested (Table 12). Thus, the HMO mixture was not genotoxic under the tested conditions at concentrations up to 60 mg/mL (28.3 mg/mL 2'-FL).

Table 12. *In vitro* Micronucleus Test in Human Peripheral Blood Lymphocytes Exposed to an HMO Mixture Containing 47.1% 2'-Fucosyllactose[b]

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

Values are means (n = 2).
CBPI = Cytokinesis block proliferation index; RI = Replicative Index.
[a]Significantly different from negative control ($p \leq 0.05$).
[b]The HMO mixture also contained 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates manufactured by Chr. Hansen A/S.

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

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

Two 90-day toxicity studies conducted in rats with 2'-FL have been published since the filing of GRN 571 (van Berlo et al., 2018; Penard et al., 2015 cited in GRN 650).

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

As summarized on pg. 31 of GRN 650, Penard et al. (2015) evaluated the toxicity of a 2'-FL manufactured by fermentation (Glycom) in an adapted 90-day oral toxicity, which involved 7-day-old neonatal Wistar [Crl:WI(Han)] rats. Either 0, 2,000, 4,000, or 5,000 mg 2'-FL/kg body weight/day was administered to 7-day-old neonatal Wistar [Crl:WI(Han)] rats via gavage for 90-days. A reference group was also included that received fructooligosaccharides (FOS) at a dose

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

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

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

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

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

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

A seven-day feeding toxicity study and two OECD-complaint 90-day toxicity studies conducted in rats with mixtures of HMOs containing 2'-FL have been published (Parschat et al., 2020; Phipps et al., 2018).

i. Seven-day Dietary Toxicity Study

In a seven-day pilot feeding toxicity study, female CD/Crl:CD rats (Charles River Laboratories, Sulzfeld, Germany) received either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Chr. Hansen A/S (n=5/group) (Parschat et al., 2020). All animals were individually housed. The HMO mixture contained 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL and 5.1% dry weight other carbohydrates, all of which were manufactured by fermentation. Thus, the overall dietary exposure to 2'-FL was 4.71 % of the diet. Both diets were provided ad libitum. Animals were observed daily for viability, behavioral changes, and reactions to treatment or illness. Cage-

side observations included skin and fur, eyes, mucous membranes, respiratory and circulatory systems, somatomotor activity, behavior patterns, and feces output and consistency. Body weight was recorded at the time of group allocation, on the 1st day of treatment, and daily thereafter at the same time each day. Feed consumption was recorded daily and feed intake per rat (g/rat/day) was calculated subtracting the total amount of feed left from the total amount of feed given and dividing the difference by the number of days and body weight of the rat. Drinking water consumption was monitored daily by visual inspection. Intake of the test article was calculated on a daily and weekly basis throughout the experimental period based on the concentration in the diet, individual feed intake, and body weight of each rat.

No mortalities occurred during the study. No HMO-related differences in behavior, appearance and consistency of the feces, body weight, body weight gain, or feed consumption were observed. Thus, the dose of 10% HMO mixture in the diet (47.1% 2'-FL by dry weight, providing 2'-FL as 4.7% of the total diet) was chosen for the subsequent 13-week dietary toxicity study in rats.

ii. Thirteen-Week Toxicity Studies

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

As summarized in GRN 921, Parschat et al. (2020) fed either a control diet (ssniff-R/M-H V1530 (ssniff Spezialdiäten, Soest, Germany)) or the same diet containing 10% of an HMO mixture manufactured by Chr. Hansen A/S to rats for 90 days (n=10/sex/group) in an OECD 408-compliant 90-day dietary toxicity study. The HMO mixture contained 47.1% dry weight 2'-FL, 16.0% dry weight 3-FL, 23.7% dry weight LNT, 4.1% dry weight 3'-SL, 4.0% dry weight 6'-SL, and 5.1% dry weight other carbohydrates, all of which were manufactured by fermentation. The overall dietary exposure to 2'-FL was 4.7% of the diet. Both diets were provided *ad libitum.* All animals were individually housed, and observed daily for clinical signs of toxicity and twice daily for mortality. Cage-side observations included changes in the skin, fur, eyes and mucous membranes, the occurrence of secretions or excretions, autonomic activity

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

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

Based on feed consumption data, the mean intake of the HMO mixture ranged from 5.01 to 6.88 g/kg bw/day for male rats and 6.26 to 7.91 g/kg bw/day for female rats. This resulted in a mean intake of 2'-FL of 2.36 to 3.24 g/kg bw/day in males and 2.95 to 3.73 g/kg bw/day in females.

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

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

Except for a statistically significant reduction ($p \leq 0.05$) in the absolute number of neutrophilic granulocytes in female rats receiving the HMO mix compared to the control

(0.71±0.38 x 10^3 vs 0.80±0.2 x 10^3 cells/µl), there were no significant differences between the control and HMO mix groups in any of the remaining hematological parameters. There were also no significant differences between the groups in the myeloid/erythroid ratio in the bone marrow.

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

Statistically significant changes were also noted in selected clinical chemistry parameters in male and female rats receiving the HMO mixture compared to the males and females receiving the control diet (Table 13). Specifically, in the HMO-treated males, significant increases in HDL-C were observed, although the levels overall were within the historical range for the laboratory and this species. In the HMO-treated female rats, plasma levels of albumin ($p \leq 0.05$), globulin ($p \leq 0.01$), total protein ($p \leq 0.01$), urea ($p \leq 0.01$), and the plasma albumin/globulin ratio ($p \leq 0.05$) were significantly increased while ALT was significantly decreased ($p \leq 0.05$) compared to the control group. All means for these parameters were within the historical range for the laboratory and the species, and not greater than 15% different from the control group means. Importantly, because the plasma albumin, globulin, protein, urea, and albumin/globulin ratio values were all within the historical range for the laboratory and the species, and small in magnitude (≤ 15%), these changes were deemed unrelated to the HMO mixture.

Table 13. Statistically Significant Differences in Clinical Chemistry Values on Day 92

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

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

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

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

Some statistically significant differences in absolute and relative organ weights were noted between control and the HMO mixture-treated groups (Table 14 and Table 15, respectively). Specifically, the absolute weight of the brains in HMO-treated male rats was lower ($p \leq 0.05$), the absolute weights of the right kidneys were lower in HMO-treated female rats ($p \leq 0.05$), and the relative weights of the left and right kidneys were lower in the HMO-treated female rats ($p \leq 0.05$). There were no significant differences in the absolute and relative weights of the other organs examined. Review of the individual animal data revealed that one female rat in the HMO-treated group had an absolute weight of the right kidney less than the lower boundary of the historical range for the laboratory. The left kidney of the same animal was also small relative to the other rats in the group (0.79 g versus a range of 0.92-1.12 g for the other female rats) and approached the lower boundary of the historical range (0.78-1.40 g). Together, these results indicated that the kidneys in this individual female were generally smaller than other rats in the HMO-treated group. None of the absolute or relative organ weight changes in the HMO-treated rats were associated with histopathologic changes. Therefore, because the brain and kidney changes were within the historical range for the laboratory, the kidney changes in the HMO group were exaggerated by a single animal with small kidneys, and the changes in the absolute and relative organ weights were not associated with adverse clinical chemistry effects or histopathologic changes, the significant differences in the absolute and relative organ weights in the HMO-treated group were deemed as biological variation.

Table 14. Significant Differences in Mean Brain and Kidney Weights

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

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

Table 15. Significant Differences in Mean Relative Kidney Weights

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

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

An uncertain test-item related histopathologic finding was present in the livers of males that had *ad libitum* access to a diet containing the HMO mix. Within the livers of 7 out of 10 males in this group, minimal to slight hepatocellular (ORO-positive) lipid content was noted in the periportal areas mainly, while only 3 males in the standard control group showed the presence of minimal ORO positive fat vacuoles. This marginal change is believed to possibly reflect a change in energy homeostasis known to be associated with an increase in sugar intake in rats (Burgeiro et al., 2017). Because females did not show such an increase and the increase in lipid content in the males was not associated with any other liver pathology, the finding was considered to be not adverse or of toxicologic relevance. No other differences in histopathological observations were observed between the HMO mixture and control groups.

Overall, no signs of toxicity were observed following the administration of an HMO mixture (containing 47.1% 2'-FL by dry weight) at 10% of the diet for 13 weeks. Based on feed intake data, the NOAEL for this study was 5.67 g/kg bw/day for male rats and 6.97 g/kg bw/day for female rats. This resulted in a mean intake of 2'-FL of 2.67 g/kg bw/day in males and 3.28 g/kg bw/day in females.

D. TOLERANCE STUDIES IN NEONATAL PIGLETS

Two published studies have evaluated the tolerance of 2'-FL in the neonatal piglet, which is an appropriate model for understanding the tolerance of a food ingredient in infants (Litten-Brown et al., 2010). Hanlon and Thorsrud (2014) evaluated the safety and tolerance of a 2'-FL manufactured by Chr. Hansen A/S, and Hanlon (2020) evaluated the safety and tolerance of a mixture of HMOs manufactured by Chr. Hansen A/S containing 2'-FL, 3'-FL, LNT, 3'-SL, and 6'-SL. The study conducted by Hanlon and Thorsrud (2014) is extensively summarized in GRN 571. The study conducted by Hanlon (2020) is extensively summarized in GRN 922. Summaries of both studies are incorporated by reference.

As summarized on pages 31 and 32 of GRN 571, Hanlon and Thorsrud (2014) administered a typical milk replacer (ProNurse® Specialty Milk Replacer) or the same typical milk replacer supplemented with 200 mg, 500 mg or 2000 mg 2'-FL/L was administered to 2-day old Yorkshire piglets for 21 days. The diets were administered via a feeding bowl that was filled six times per day at a dose volume of 500 mL/kg/day or 8.33 mL/kg/dose. All piglets survived to scheduled necropsy on Day 22. There were no reported dose-responsive adverse clinical findings during the dosing period. Both male and female piglets showed good growth based on body weight gain and feed efficiency. There were no reported treatment-related adverse effects on the clinical pathology parameters evaluated, including hematology, clinical chemistry, coagulation and urinalysis. There were no reported treatment-related adverse macroscopic and microscopic findings, including intestinal pH. The microscopic findings included mild to moderate inflammation within the keratinized portion of the squamous epithelium in the non-glandular part of the stomach of one male and one female in the 2000 mg/L group and in one female in the 500 mg/L dose group. The one male in the 2000 mg/L group also showed focal loss/thinning in the keratinized portion of the squamous epithelium, associated with inflammation but without ulceration. There were no macroscopic findings associated with the observation. All other microscopic findings were considered incidental and were within the range of typical observations in swine of this age and strain. The authors concluded that the daily dietary administration of Chr. Hansen A/S 2'-FL to neonatal piglets for three weeks following birth 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.

As summarized on pages 38 – 70 in GRN 921, Hanlon (2020) administered a mixture of HMOs containing 2'-FL, 3'-FL, LNT, 3'-SL, and 6'-SL to two-day-old Yorkshire crossbred piglets for 21 days. Thirty-six experimentally naïve domestic two-day-old Yorkshire crossbred piglets were assigned to one of three treatment groups (n=12/group). The treatment groups received either a control diet, a diet containing 5.75 g/L of HMO MIX 1, or a diet containing 8.0 g/L HMO MIX 1. The control diet was Land O'Lakes Specialty Milk Replacer and was used as

the base diet for both HMO Mix 1 test diets. HMO MIX 1 was obtained from Chr. Hansen A/S (Rheinbreitbach, Germany) and contained 49.1% 2'-FL, 10.4% 3-FL, 19.9% LNT, 3.5% 3'-SL, and 4.2 % 6'-SL on a dry weight basis. The endpoints that were evaluated included mortality, clinical observations, body weight, feed consumption, feed efficiency, compound consumption, clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), gross necropsy findings, organ weights, and histopathologic examinations. Except for one male piglet in the 8.0 g/L dosing group, which was euthanized on day 7 for humane reasons, all of the remaining animals survived until the scheduled study termination on day 22. The clinical and veterinary observations of the male piglet in the 8.0 g/L dosing group that was euthanized included yellow discolored feces, thin body condition, unkempt appearance, generalized muscle wasting, and lateral recumbency. Additionally, *E. coli* was detected in a fecal culture of the one male piglet that was euthanized. Based on the presence of *E. coli* in the feces and the constellation of observations, the unscheduled death/euthanasia of the one male in the 8.0 g/L treatment group was determined to be not HMO Mix 1-related, but rather due to an underlying infection that was distributed evenly among the animals in all dosing groups. The clinical pathology values, and macroscopic and microscopic findings in the remaining animals did not reveal a relationship to the HMO Mix 1 treatment and, although increased cecum weights in males and females at ≥5.75 g/L, increased colon weights in males at ≥5.75 g/L, and decreased rectum weights in males and females at 8.0 g/L were observed, these changes were considered not adverse as there were no microscopic correlates. Importantly, the underlying infection did not affect the validity of the results. Together these results indicate that daily dietary administration of HMO Mix 1 to neonatal piglets for 3 weeks at concentrations up to 8.0 g/L with calculated intakes of 3.6 and 3.7 g/kg/bw (1.8 and 1.8 g 2'-FL/kg bw) in males and females, respectively, was well-tolerated, did not produce adverse effects on growth and development. Since the filing of GRN 921, this study was published by Hanlon (2020).

E. CORROBORATIVE ANIMAL STUDIES

Recently, a corroborative study was published by Chleilat et al. (2020) where 3-week-old Sprague Dawley rats were fed either a control diet or diets containing either 0.625% 2'-FL, 0.625% 3'-SL, or 0.625% 2'-FL and 0.625% 3'-SL for eight weeks. Body composition, intestinal permeability, serum cytokines, fecal microbiota composition, and messenger RNA (mRNA) expression of selected genes involved in gut barrier function in the gastrointestinal tract (5 males and 5 females/group) were assessed after the 8-week treatment period. There were no differences in body composition among the groups. Males in the HMO-fed groups had a small, but significant decrease in body weight at week 8 of the study (p=0.03), as well as significantly lower levels of the proinflammatory cytokine interleukin 18 (IL-18) in their serum (p=0.01). Female rats fed the diet containing both 2'-FL and 3'-SL had significantly increased cecum weight compared to the control (p=0.002), and significantly decreased colon weight compared to

the control (p=0.03) and the 3'-SL fed groups (p=0.02). All females fed HMOs had significant reductions in intestinal permeability compared to controls whereas no significant differences were observed among the different male groups. All HMO-fortified diets also altered gut microbiota composition and mRNA expression in the gastrointestinal tract, albeit differently according to sex. Importantly, the authors concluded that supplementation with a fraction of the HMOs found in human breast milk has a complex sex-dependent risk/benefit profile. The weight of the evidence reported in this study suggests that HMO supplementation in general has functional benefits, such as lowering proinflammatory cytokine gene expression and reducing intestinal permeability. Additionally, the increase in cecum weight reported in this study is consistent with the results of other studies that have administered non-digestible carbohydrates to rats for extended periods of time (Zhou et al., 2017; Adam et al., 2015; Konishi et al., 1984; Oku et al., 1998; Nzeusseu et al., 2006; Jacobs and Schneeman, 1981).

F. CLINICAL STUDIES

Additional support for the safe use of 2'-FL in toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas at the intended use level is based on results of numerous clinical studies that evaluated the safety and tolerance of HMOs, including 2'-FL, as well as other non-digestible carbohydrates in infants, adults, sensitive populations consuming enteral tube feeding formulas and oral electrolytes solutions. In general, HMOs are well tolerated in infants up to 1 g/day, adults up to 20 g/day and non-digestible carbohydrates are well tolerated in enteral tube feeding formulas up to 63 g/day and oral electrolyte solutions up to 50 g/L.

1. Clinical Studies with HMOs in Infants and Adults

2'-Fucosyllactose is a non-digestible HMO that is GRAS for use in infant formula, conventional foods, and enteral formulas (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019; GRN 897, 2020). Since the filing of GRN 571, eleven clinical studies conducted by Marriage et al. (2015), Goehring et al. (2016), Elison et al. (2016), Storm et al. (2019), Nowak-Wegrzyn et al. (2019), Puccio et al. (2017), Kajzer et al. (2016), Riechmann et al. (2020), Iribarren et al. (2020), Palsson et al. (2020), and Ryan et al. (2021) have been published that evaluated safety and tolerance of 2'-FL-supplemented infant formulas and foods. In infants, Storm et al. (2019) administered 2'-FL alone whereas Marriage et al. (2015), Goehring et al. (2016), Nowak-Wegrzyn et al. (2019), Puccio et al. (2017), Kajzer et al. (2016), and Riechmann et al. (2020) administered mixtures of oligosaccharides containing 2'-FL. In adults, Elison et al. (2016), Palsson et al., 2020, Iribarren et al. (2020), and Ryan et al. (2021) administered either 2'-FL, lacto-N-neotetraose (LNnT), or a mixture of 2'-FL and lacto-N-neotetraose (LNnT). Other clinical studies have also been conducted with the acidic HMOs 3'-SL and 6'-SL (Simeoni et al., 2016; Cooper et al., 2016; Radke et al., 2017; Rasko et al., 2000;

Parente et al., 2003; Gurung et al., 2018). Except for the studies conducted by Riechmann et al. (2020), Iribarren et al. (2020), Palsson et al. (2020), and Ryan et al. (2021), all of these studies have been extensively summarized in previous GRAS Notices (GRN 546, 2015; GRN 571, 2015; GRN 571 Supplement, 2019; GRN 650, 2016; GRN 659, 2016; GRN 735, 2018; GRN 749, 2018; GRN 766, 2018; GRN 815, 2019; GRN 852, 2019; GRN 880, 2020; GRN 897, 2020; GRN 919, 2020; GRN 921, 2020) and therefore their summaries are incorporated by reference and the studies are briefly re-summarized in tabular format below along with the new studies conducted by Riechmann et al. (2020), Iribarren et al. (2020), Palsson et al. (2020), and Ryan et al. (2021) (Tables 16 and 17).

In infants, Storm et al. (2019), Marriage et al. (2015), Goehring et al., (2016), Puccio et al. (2017), Nowak-Wegrzym et al. (2019), and Riechmann et al. (2020) administered up to 1.0 g 2'-FL/L and 0.5 g LNnT/L to infants (equivalent to approximately 1.0 g 2'-FL/day and 0.5 g LNnT/day, assuming that infants consume one liter of formula day) and reported that both HMOs were well-tolerated and had no adverse effect on growth and development (Table 16). Meli et al. (2014), Simeoni et al. (2016), Cooper et al. (2016), and Radke et al. (2017) reported similar effects when a mixture of oligosaccharides containing 3'-SL, galactooligosaccharides, and 6'-SL up to a total of 10 g total oligosaccharides/L were administered (equivalent to approximately 10 g total oligosaccharides /day, assuming that infants consume one liter of formula day), although the levels of 3'- SL and 6'-SL ingested in the studies were not provided in the publications (Table 16). Importantly, none of the studies reported serious adverse events related to the ingestion of the HMOs and the most common effects were occasional flatulence, abdominal distress, diarrhea, and loose stools, which are not unexpected considering what is known to occur following the ingestion of diets containing high amounts of non-digestible carbohydrates (Eldridge et al., 2019).

In adults, Elison et al. (2016), Iribarren et al. (2020), Palsson et al. (2020), and Ryan et al. (2021) showed that the ingestion of up to 20 g/day of either 2'-FL, LNnT, or a combination of 2'-FL and LNnT in healthy adults and adults with inflammatory bowel disease (IBS), ulcerative colitis, Crohn's disease, or celiac disease was well tolerated and as expected, the most common complaints were flatulence, abdominal distress, and abdominal pain. Similar results were also reported by Rasko et al. (2000), Parente et al. (2003), and Gurung et al. (2018) when the subjects ingested 20 g 3'-SL/day (Table 17).

Table 16. Clinical Studies with Human Milk Oligosaccharides and Infants

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
			2'-Fucosyllactose		
Riechmann et al., 2020	Non-randomized, open-label, prospective study Healthy term infants 7 days to 2 months old	Group 1: Formula-fed infants (n=82) Group 2: Infants consuming formula and human milk; the formula contained 1.0g/L of 2'-FL, 0.5 g LNnT, and Lactobacillus reuteri (n=62) Group 3: Breast-fed infants (n=63)	8 weeks	• Sixteen subjects dropped out of Group 1 (six were excluded due to protocol deviations, three dropped out due adverse events (AEs), and seven were lost to follow-up). • Fourteen subjects dropped out of Group 2 (eight were excluded due to protocol deviations, 3 dropped out due to adverse events, and three were lost to follow-up. • Eighteen subjects dropped out of Group 3 (11 were excluded due to protocol deviations, one dropped out due to adverse events, and 6 were lost to follow-up. • There were no significant differences between any of the groups for any of the anthropometric measures. • Composite Infant Gastrointestinal Symptom Questionnaire (IGSQ) scores demonstrated low gastrointestinal distress in all feeding groups at all time points and there were no significant differences among feeding groups at baseline, 4, or 8 weeks. o There were no significant differences among the groups in the gassiness, fussiness, crying or spitting-up/vomiting domains of the IGSQ. o For the stooling domain, Group 2 were significantly different than Group 3 at baseline and 8 weeks. • A total of 49 subjects experienced 58 adverse events over the course of the study. There were 19 AEs in Group 1, 21 in Group 2, and 18 AEs in Group 3. The incidence was generally low and not significantly different among the groups o Three subjects experienced potentially product-related AEs, including two instances of cow's milk intolerance (one in Group 1 and one in Group 2) and one instance of irritability in Group 1. • Six serious adverse events occurred (four in Group 1 and 2 in Group 2), all of which were bronchiolitis. All were considered unrelated to the study feeding.	Not previously summarized
Nowak-Wegrzyn et al., 2019	Double-blind, placebo-controlled food challenges	Treatment #1: Whey-based extensively hydrolyzed formula Treatment #2: Whey-based extensively hydrolyzed	Not applicable	• Sixty-four children completed at least one DBPCFC. • Three children were excluded due to protocol deviations (n = 61). • There was one allergic reaction to the Test, and one to the Control formula. • Sixty-one of the 64 subjects completed the open-label home challenge phase with the Test formula	GRN 919, page 33

Table 16. Clinical Studies with Human Milk Oligosaccharides and Infants

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
	Children with cow milk protein allergy	formula containing 1.0 g/L 2'-FL and 0.5 g/L LNnT		○ One subject vomited on Day 1 of the home challenge but completed the home challenge without further problems. ○ One patient developed diarrhea on the last day of the challenge, which the site investigator attributed to gastroenteritis. ○ No significant gastrointestinal symptoms (flatulence, abnormal stool frequency/consistency, increased spitting-up, or vomiting) were reported. • No serious adverse events occurred during the entire study.	
Storm et al., 2019	Randomized, placebo-controlled double-blind study Healthy term infants 14 days old ±5 days.	Group 1: Formula containing *Bifidobacterium animalis* ssp *lactis* Bb12 (n=40) Group 2: Formula containing *Bifidobacterium animalis* ssp *lactis* Bb12 + 0.25 g/L 2'-FL (n=38)	6 weeks	• In the 2'-FL-treated group, one subject was lost to follow-up, one caregiver wished to withdraw, three subjects withdrew due to adverse events (AEs), and three subjects did not comply with feeding only the study formula. • In the control group, one subject was lost to follow-up, one caregiver wished to withdraw, three subjects withdrew due to adverse events, and two subjects did not comply with feeding only the study formula. • Infant gastrointestinal symptom questionnaire scores were similar in both groups at baseline and after 6 weeks of treatment. • Stool frequency and consistency did not differ between the groups over the course of treatment. • Significantly more stools were reported to be difficult to pass in the control group than in the test group ($p<0.05$), however, the number of infants with stools reported as difficult to pass was not different between the groups. • Crying, fussing duration, vomiting frequency, and the proportion of babies reported to have any spit up over the 2-day diary period were similar between the two groups. • Among the babies whose caregivers reported spit-up, significantly more were reported to have spit up >5 times/day in the 2'-FL group compared to the control group. • There were no serious AEs and the AEs were equally distributed among the two groups. • There were significantly more subjects that experienced infections and infestations in the control group than in the 2'-FL-treated group (n=9 vs n=3; p=5). • There were no effects of the 2'-FL-containing formula on anthropometric measures (body weight and lengths, and weight-for-age and length-for-age).	GRN 571 supplement, page 21

Table 16. Clinical Studies with Human Milk Oligosaccharides and Infants

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
Puccio et al., 2017	Prospective, randomized, placebo-controlled study Healthy, term infants 0 to 14 days old	Group 1: Formula (n=87) Group 2: Formula with 1.0 g/L 2'-FL and 0.5 g/L LNnT (n=88)	6 months (after 6 months, all infants were switched to a non-HMO containing formula)	• Twenty infants in control and 24 infants in the HMO containing formula withdrew before the primary outcome assessment at 4-months. The dropout rate was comparable between groups. The most common reason for discontinuation was an adverse event (n=11 in control; n=12 in test). Other reasons for discontinuation before 4 months included parent/guardian request (n=3 in control; n=6 in test); lost to follow-up/missing (n=5 in control; n=6 in test); and other (n=1 in control; n=40 in test). • There was no difference in weight gain, mean weight-for-age, length-for-age, head circumference-for-age, and BMI-for-age z scores between the groups. • Parent-reported infant behavioral patterns including restlessness/irritability and colic were similar in the HMO and control groups except for softer stool (P=0.021) and fewer nighttime wake-ups (P = 0.036) in the test group at 2 months. • Infants receiving the HMO-containing formula had significantly fewer parental reports (P = 0.004 – 0.047) of bronchitis through 4 (2.3% vs 12.6%), 6 (6.8% vs 21.8%), and 12 months (10.2% vs 27.6%); lower respiratory tract infection (adverse event cluster) through 12 months (19.3% vs 34.5%); antipyretics use through 4 months (15.9% vs 29.9%); and antibiotics use through 6 (34.1% vs 49.4%) and 12 months (42.0% vs 60.9%) compared to the infants receiving the control formula.	GRN 650, page 38
Goehring et al., 2016	Prospective, randomized, placebo-controlled study Healthy, term infants 5 days old	Group 1: Formula with GOS (n=39) Group 2: Formula with GOS + 0.2 g/L 2'-FL (n=37) Group 3: Formula with GOS + 1.0 g/L 2'-FL (n=37) Group 4: human milk (HM)(n=42)	16 weeks	• Note: This is a sub-study of the clinical study conducted by Marriage et al., 2015. The objective was to investigate the effects of feeding formulas supplemented with HMO 2'-FL on biomarkers of immune cell function. • Circulating plasma concentrations of inflammatory cytokines IL-1a, IL-1b, IL-6, and TNF-a and anti-inflammatory IL-1ra were significantly higher (82%, 72%, 76%, 58%, and 58%, respectively) in the group fed formula compared to the group receiving human milk ($p \leq 0.05$). • Both the groups receiving the formulas containing 2'-FL exhibited profiles that were significantly different from the formula group and not different from the human milk group or each other. There were no differences in plasma cytokines IFN-a2, IFN-g, IL-10, IP-10, or RANTES between any of the groups.	GRN 735, page 62

Table 16. Clinical Studies with Human Milk Oligosaccharides and Infants

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
Marriage et al., 2015	Prospective, randomized, placebo-controlled study Healthy, term infants 5 days old	Group 1: Formula with GOS (n=101) Group 2: Formula with GOS + 0.2 g/L 2'-FL (n=104) Group 3: Formula with GOS + 1.0 g/L 2'-FL (n=109) Group 4: human milk (HM)(n=106)	17 weeks	• 338 infants completed the study (84 in the control group, 81 in the group receiving the formula containing 0.2 g/L 2'-FL, 83 in the group receiving the formula containing 1.0 g/L 2'-FL, and 90 in the HM group); 304 of whom completed the study on the assigned feeding or HM (79 in the control group, 70 in the group receiving the formula containing 0.2 g/L 2'-FL, 72 in the group receiving the formula containing 1.0 g/L 2'-FL, and 83 in the HM group). The number of premature terminations was not statistically significant among the formula-fed groups. • Although the HM group gained significantly more weight than the group receiving 0.2 g/L 2'-FL from day 14 to 28 and the group receiving 1.0 g/L 2'-FL than the HM group from day 84 to 119, there were no significant differences (sex-specific or sex- combined) in mean weight, length, or head circumference among feeding groups during the study, and no significant differences among feeding groups in mean gains in these measures from day 14 to 119. • The mean number of stools/day was significantly higher for the HM group compared to all groups receiving the formulas for the three days before the study visits at day 28, 42, and 84. The mean number of stools/day was also significantly higher for the HM group compared to the control formula group for the three days before the study visits at day 119. • Although spitting-up or vomiting was significantly higher in the formula-fed groups compared to the HM group from enrollment to day 28, there were no differences after day 28. • Although the mean rank stool consistency was significantly higher for the group receiving 2'-FL from enrollment to day 28 and was significantly higher in the formula-treated groups than the HM group for the remainder of the study, there was no difference among the formula-treated groups over the course of the study. • There were no significant differences in the overall percentage of subjects experiencing adverse events or serious adverse events in the formula-treated groups. • The control formula and the 1 g/L 2'-FL groups had significantly more subjects with reported adverse events in the "infections and infestations" category compared with the 0.2 g/L group ($p<0.05$), but the types of adverse events were similar (upper respiratory tract symptoms; otitis media, viral infections, and oral candidiasis. The control formula-treated group also had a significantly higher percentage of subjects with eczema ($p<0.05$)	GRN 650, page 37

Table 16. Clinical Studies with Human Milk Oligosaccharides and Infants

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
Kajzer et al., 2016 (abstract)	Prospective, randomized, double-blind, placebo-controlled study Healthy term infants 0 and 8 days of age.	Group 1: Formula (n=42) Group 2: Formula with 0.2 g/L 2'-FL and 2 g/L scFOS (n=46) Group 3: human milk (HM)(n=43)	5 weeks	• Thirty-six (86%) subjects in the group receiving formula, 41 (89%) in the group receiving oligosaccharides and 42 (98%) in the group receiving human milk completed the study. • There was no difference in the mean rank stool consistency among the groups. • The average number of stools per day for the human milk group was significantly higher in the human milk group than both formula-fed groups. • There were no differences among groups for the average volume of study formula intake, number of study formula feedings/day, anthropometric data or percent feeding with spit-up/vomit. • Safety endpoints not reported.	GRN 571, page 21
Alliet et al., 2016 (abstract)	Randomized, placebo controlled, study Healthy term infants 0-14 days old	Group 1: Cow's milk-based infant formula (n=87) Group 2: Cow's milk-based infant formula w/ 1.0 g/L 2'-FL and 0.5 g/L LNnT (n=88) Group 3: Human milk	3 months	• 2'FL and LNnT shift the stool microbiota towards that observed in breastfed infants. • Safety endpoints not reported.	GRN 815, page 55
Steenhout et al., 2016 (abstract)	Randomized, placebo controlled, study Healthy term infants 0-14 days old	Group 1: Cow's milk-based infant formula (n=87) Group 2: Cow's milk-based infant formula w/ 1.0 g/L 2'-FL and 0.5 g/L LNnT (n=88) Group 3: Human milk	3 months	• 2'FL and LNnT shift the stool microbiota towards that observed in breastfed infants. • Safety endpoints not reported.	GRN 735, page 62

Table 16. Clinical Studies with Human Milk Oligosaccharides and Infants

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
3'-Sialyllactose and 6'-Sialyllactose					
Radke et al., 2017	Multicenter, randomized placebo-controlled, double-blind study Healthy term infants 0-14 days old	Group 1: Control formula; (n=207) Group 2: Test formula containing 5.8 ± 1.0 g BMOs*/100 g powdered formula (8 g/L in the reconstituted formula) and $1x10^7$ cfu/g *B. lactis* CNCM I-3446; (n=206) Group 3: Breastfed reference group; (n=63) *BMOs were generated from whey permeate and contained galactooligosaccharides and milk oligosaccharides, such as 3'- and 6'- sialyllactose; the concentrations of 3'- and 6'- sialyllactose are not known	6 months Follow-up at 12 months, no test formula 6-12 months	• A total of 58 infants (27 in each of the Test and the Control groups and four in the Breast-fed group) were excluded from the ITT analyses because they dropped out before the 1-mo visit. • The population that completed the entire study duration was 150 infants in the test formula group, 157 in the control formula group, and 49 in the breastfed group. • The proportion of infants with AEs related to infections was comparable among the formula groups. • No significant difference in diarrhea or febrile infections incidence among the groups at 6 and 12 months. • Test formula was well tolerated and no difference in anthropometric measures were observed among the groups. • The test formula group showed similar gut microbiota patterns, fecal IgA, and stool pH to breastfed infants and was significantly different than the control formula group.	GRN 766, pages 62-64
Simeoni et al., 2016	Randomized, placebo-controlled, double-blind study Healthy 5-day old, term infants	Group 1: Standard formula; (n=37) Group 2: Standard formula plus 5.7±1.0 g/100 g bovine milk oligosaccharides (BMOs*; 8.0 g/L reconstituted formula) and $1x10^7$ cfu/g of *B. lactis* CNCM I-3446; (n=39) Group 3: Human milk; (n=37)	12 weeks	• No difference in compliance or tolerability was observed among the three groups. o 10 infants discontinued in the human milk/breastfed group (5 withdrew voluntarily and 5 for other reasons) o 7 infants discontinued in the standard formula group (2 withdrew due to GI symptoms, 4 withdrew voluntarily, and 2 were lost to follow-up o 7 infants discontinued in the standard formula with the BMOS and *B. lactis* CNCM I-3446 group (3 withdrew due to GI symptoms, 2 withdrew voluntarily, and 3 were lost to follow-up • There were no differences in anthropometric measures among the three groups.	GRN 766, pages 62-64

Table 16. Clinical Studies with Human Milk Oligosaccharides and Infants

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
		*BMOs were generated from whey permeate and contained galactooligosaccharides and milk oligosaccharides, such as 3'- and 6'- sialyllactose; the concentrations of 3'- and 6'- sialyllactose are not known		• There were no differences in the standard formula and standard formula with BMOS and *B. lactis* CNCM I-3446 groups in 'spitting up', vomiting, crying, colic, flatulence and irritability. • Infants from the standard formula with BMOS and *B. lactis* CNCM I-3446 group, but not the standard formula only group, showed a proportion of yellowish versus greenish stools equivalent to the breast-fed infants. • Infants in the standard formula with BMOS and *B. lactis* CNCM I-3446 group showed more liquid stools than infants in the standard formula group; liquid stools were the dominant observation in the breast-fed infants.	
Cooper et al., 2016	Multicenter, randomized, placebo-controlled, double-blind study Healthy term infants born to HIV+ mothers	Group 1: Cesarean-delivered infants consuming standard formula; (n=101) Group 2: Cesarean-delivered infants and standard formula containing 5.8 ± 1.0 g BMOs*/100 g powder formula (8 g/L in the reconstituted formula) and $1x10^7$ cfu/g *B. lactis* CNCM I-3446; (n=92) Group 3: Vaginally delivered infants and standard formula; (n=113) Group 4: Vaginally delivered infants standard formula containing 5.8 ± 1.0 g BMOs/100 g powder formula (equivalent to 8 g/L in the reconstituted formula) and $1x10^7$ cfu/g *B. lactis* CNCM I-3446; (n=115)	4 months	• Four hundred and thirty infants were randomized into the study. o Nine (2.1%) infants were lost to follow-up after randomization but before starting the study formulas. o Eight infants were found to be HIV infected, seven at the 4-week visit (v2) and one became positive at 6 months (v5). o Of the eight that were HIV infected, three infants died and one discontinued the study. o Over the course of the study, there were a total of 55, 57, 47, and 55 discontinuations in the vaginal starter formula containing BMOs and *B. lactis* CNCM I-3446, vaginal group starter formula, cesarean starter formula containing BMOs and *B. lactis* CNCM I-3446, and cesarean starter formula groups, respectively. o There were no significant differences in tolerability and adverse events between the groups in both delivery methods. • Test formula supplemented with BMOS lowered fecal pH and improved fecal microbiota counts in both delivery methods.	GRN 766, pages 62-64

Table 16. Clinical Studies with Human Milk Oligosaccharides and Infants					
Reference	**Study Design and Population**	**Groups (Numbers of Subjects)**	**Duration**	**Safety Parameters**	**GRN Reference**
		*BMOs were generated from whey permeate and contained galactooligosaccharides and milk oligosaccharides, such as 3'- and 6'- sialyllactose; the concentrations of 3'- and 6'- sialyllactose are not known			
Meli et al., 2014	Randomized, double-blind, single-center study Healthy term infants (<14 days old)	Group 1: Standard formula; (n=84) Group 2: Standard formula plus 10 g bovine milk oligosaccharides (BMOs*/L); (n= 99) Group 3: Standard formula plus 10 g BMOs/L, 2×10^7 cfu/g *Bifidobacterium longum* ATCC BAA-999 (Bl999), and 2×10^7 cfu/g *Lactobacillus rhamnosus* CGMCC 1.3724 (LPR); (n=98) Group 4: Human milk; (n=39) *BMOs were generated from whey permeate and contained galactooligosaccharides and milk oligosaccharides, such as 3'- and 6'- sialyllactose; the concentrations of 3'- and 6'- sialyllactose are not known	4 months	• 90 infants from formula groups and 18 infants from breastfed groups withdrew o Higher rates of discontinuations were observed in the BMOS-supplemented formula groups (36.4% in Group 2; 34.7% in Group 3) compared with the standard formula-treated group (23.8%), although the differences did not reach statistical significance. o GI symptoms (i.e., regurgitation, vomiting, diarrhea, constipation, and abdominal pain characterized by prolonged crying) were the most common reason for study discontinuation in all three formula groups: 14.3% of infants in the standard formula-treated group, 17.2% in Group 2 and 13.3% in the Group 3 discontinued due to GI symptoms. • Weight gain and length and head circumference showed no significant differences between standard and BMOS-containing formula groups • BMOS groups had more frequent and less hard stools compared to control • No significant differences were observed between the standard and BMOS containing formula-treated groups in caregivers' reports of flatulence, vomiting, spitting up, crying, fussing, and colic.	GRN 766, pages 62-64

Table 17. Clinical Studies with Human Milk Oligosaccharides and Adults

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
2'-Fucosyllactose and/or Lacto-N-neotetraose					
Ryan et al., 2021	Open-label, single arm study Adults (21 – 75 years old) with a BMI of 19-40 kg/m2 and with previously diagnosed inflammatory bowel disease (IBS), ulcerative colitis, Crohn's disease, or celiac disease	Group 1: 4 g of 2'-FL in combination with micronutrients, macronutrients, amino acids, and isomalto-oligosaccharide (n=20)	6 weeks	• Twelve subjects completed the study. • Eight subjects withdrew from the study o Two dropped out/declined to participate o Three dropped out due to non-serious adverse events. They reported worsening of pre-existing gastrointestinal symptoms, gastrointestinal upset, and a non-study-related viral infection • Three were lost to follow-up.	Not previously reviewed
Palsson et al., 2020	Open-label, single arm study Adult male and female patients (18 and older) with IBS	Group 1: 5 g of 2'-FL/LNnT (4:1 ratio) (n=317)	12 weeks	• Thirteen subjects were discontinued after completing the baseline survey because they did not start the intervention. Therefore, 273 patients completed the study. o Eight subjects withdrew due to an adverse event. o Four subjects withdrew consent. o Nineteen subjects were lost to follow-up. • The authors reported that there were no incidents causing safety concerns and the patients generally reported that the intervention was well-tolerated o Forty-seven patients reported a total of 87 adverse events (AEs) in the study o Sixty-one of the AEs were related to the gastrointestinal tract. o The most common side effect was passing gas, followed by abdominal distension and pain. • One serious AE occurred (hospitalization due to colitis) but was determined to be unrelated to the intervention by the study's medical safety officer.	Not previously reviewed

Table 17. Clinical Studies with Human Milk Oligosaccharides and Adults

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
Iribarren et al., 2020	Parallel, double-blind, randomized, placebo-controlled study Adult male and female patients (18 – 64 years old) with inflammatory bowel syndrome (IBS).	Group 1: Placebo (n=21) Group 2: 5 g 2'-FL/LNnT (4:1 ratio) (n=20) Group 3: 10 g 2'-FL/LNnT (4:1 ratio) (n=20)	4 weeks of treatment followed by a 4-week washout	• Group 1: one patient discontinued intervention due to worsening of symptoms during the treatment period; one patient was lost to follow-up during the washout period. • Group 2: no patients left the study • Group 3: one patient discontinued intervention due to worsening of symptoms during the treatment period; one patient was lost to follow-up during the washout period. • There were no differences in overall gastrointestinal symptom severity among the groups at week four or week eight. • None of the treatments aggravated the IBS symptoms. • There were no significant differences among the groups in the individual domains of the Gastrointestinal Symptom Rating Scales (abdominal pain, bloating, constipation, diarrhea, and satiety). • Within the groups: o There was a decrease in the severity of bloating and diarrhea in Group 1 at week 4. o In Group 2 and 3, there was a decrease in bloating and abdominal pain at week 8, respectively. • There were no differences between groups or within the groups at week 4 or 8 regarding IBS symptom severity.	Not previously reviewed

Table 17. Clinical Studies with Human Milk Oligosaccharides and Adults

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
Elison et al., 2016	Randomized, placebo-controlled double-blind study Healthy male and female adults ages 18 to 60 years.	Group 1: 2g glucose (n=10) Group 2: 5 g 2'-FL (n=10) Group 3: 10 g 2'-FL (n=10) Group 4: 20 g 2'-FL (n=10) Group 5: 5 g LNnT (n=10) Group 6: 10 g LNnT (n=10) Group 7: 20 g LNnT (n=10) Group 8: 3.3 g 2'-FL; 1.7 g LNnT (n=10) Group 9: 6.7 g 2'-FL; 3.4 g LNnT (n=10) Group 10: 13.3 g 2'-FL; 6.7 g LNnT (n=10)	1-2 week run-in period followed by a 2 week treatment period	• All subjects were compliant and completed the study according to the protocol without any dropouts. • Fifty-six adverse events were reported by forty-four subjects. o All were judged as 'mild', and all subjects tolerated the investigational products throughout the trial period. o Adverse events were usually reported as a complex of multiple symptoms such as flatulence, bloating and constipation, and were primarily reported at the end of the 2-week intervention. o Most adverse events were reported by subjects taking the highest doses of 2′FL and LNnT. Gas/ flatulence was the most common adverse event reported, followed by stomach pain, diarrhea/loose stools and rumbling, but at lower frequencies. • No significant difference in bowel movement was observed compared to Group 1. • No change in clinical significance in any physical parameter including pulse rate and blood pressure was found during the 2-week intervention. • There was no difference in clinical chemistry or hematology among the groups at the end of the 2-week intervention period	GRN 735, page 61

Table 17. Clinical Studies with Human Milk Oligosaccharides and Adults

Reference	Study Design and Population	Groups (Numbers of Subjects)	Duration	Safety Parameters	GRN Reference
			3'-Sialyllactose		
Gurung et al., 2018	Randomized, double-blind, placebo-controlled study Adults with *H. pylori* infection	Group 1: Placebo (n=17) Group 1: 12 g/day 3'-SL (n=24)	4 weeks	• There were no significant differences between pre- and post-dose gastrointestinal tolerance and clinical chemistry (serum biochemistry, hematology, and urine analysis) outcomes. • Pre- and post-dose urea breath test values were not significantly different within or between the 3'-SL and placebo groups. • Compliance and adverse events were similar between the groups.	GRN 880, pages 35,36
Parente et al., 2003	Randomized, double-blind, placebo-controlled study Adults with *H. pylori* infection (dyspepsia)	Group 1: Placebo (n=21) Group 2: 10 g/day 3'-SL sodium salt (n=17) Group 3: 20 g/day 3'-SL sodium salt (n=22)	4 weeks	• Five patients were excluded from analysis due to protocol violation. • Adverse events recorded in 6 patients were halitosis, asthenia, epigastric pain, and headache. • One patient dropped out due to headache associated with epigastric pain. • No serious adverse events were observed. • *H. pylori* colonization documented by the ^{13}C-Urea Breath Test (UBT) decreased significantly (*p*-value not provided) in both treatment groups and placebo but was most likely due to regression toward mean effect.	GRN 766, pages 64-67
Rasko et al., 2000	Randomized, double-blind, placebo-controlled study Adults with *H. pylori* infection	Group 1: Placebo (n=6) Group 2: 4g 3'-SL (n=6) Group 3: 8g 3'-SL (n=7) Group 4: 20g 3'-SL (n=7)	56 days for Control and Groups 1 and 2 28 days for Group 3	• Oral supplementation of 3'-SL did not change Lewis antigen expression of *H. pylori* strains isolated from human gastric mucosa. • No adverse effects on safety or tolerance were reported.	GRN 766, pages 64-67

2. Clinical Studies with Other Non-digestible Carbohydrates and Enteral Tube Feeding Formulas

Enteral tube feeding is indicated in any patient that has a functioning and accessible gastrointestinal tract and cannot meet their nutritional requirements by consuming food orally (reviewed in Wireko and Bowling, 2010). Enteral tube feeding is administered either as a bolus or continuously via nasogastric tubes, nasojejunal tubes, or gastrostomy and can be associated with issues with the tubes and their insertion, as well as adverse effects in the patient, such as diarrhea, constipation, nausea, and vomiting/aspiration/reflux, bloating, refeeding syndrome and various electrolyte disturbances (https://gi.org/topics/enteral-and-parenteral-nutrition/; accessed on February 11, 2021). As a result, enteral tube feeding is generally administered and managed in a medical setting. Importantly, the purpose of using non-digestible carbohydrates in enteral tube feeding formulas is to help alleviate alterations in bowel function and maintain the healthy balance of the microbiota.

Although no clinical studies have been conducted with enteral tube feeding formulas containing 2'-fucosyllactose, published clinical studies administering other non-digestible, poorly absorbed carbohydrates in enteral tube feeding formulas are relevant to understanding the tolerance of 2'-FL as a non-digestible carbohydrate in enteral tube feeding formulas. As summarized in an amendment to GRN 897 to support the safe use of 2'-FL in enteral formulas, numerous published clinical studies have administered non-digestible carbohydrates, such as partially hydrolyzed guar gum (PHGG), galactomannan, fructooligosaccharides (from short-chain FOS to long-chain inulin), galactooligosaccharides (GOS), and GOS/FOS blends in enteral formulas to infants, children, healthy adults, bed-ridden elderly adults, and patients hospitalized for a variety of serious medical conditions (Akatsu et al., 2016; Alam et al., 2000; Alam et al., 2005; Armanian et al., 2016; Fussell et al., 1996; Garleb et al., 1996; Homann et al., 1994; Homann et al., 2004; Karakan et al., 2007; Khoshoo et al., 2010; Lampe et al., 1992; Meier et al., 1993; Modi et al., 2010; Nakao et al., 2002; Peters and Davidson, 1996; Rushdi et al., 2004; Simakachorn et al., 2011; Spapen et al., 2001; van den Berg et al., 2015; Zheng et al., 2006). Because these studies are summarized in the amendment to GRN 897, their summaries are therefore incorporated by reference and briefly summarized in tabular format below (Table 18). Collectively these studies show that the use of non-digestible carbohydrates in enteral tube feeding formulas at levels up to 63 g/day are well-tolerated.

Additionally, the Institute of Medicine evaluated the potential adverse effects associated with overconsumption of non-digestible carbohydrates such as PHGG, FOS, and GOS, and concluded that although occasional adverse gastrointestinal symptoms can occur (flatulence, abdominal distress, and diarrhea), serious chronic adverse effects have not been observed. Additionally, due to the bulky nature of these substances, excess consumption is likely to be self-limiting. Thus, the Institute of Medicine has not set an tolerable upper limit (UL) for individual fibers (Eldridge et al., 2019).

Taken together, these data indicate that the risk of adverse effects from the judicious use of non-digestible carbohydrates, such as 2'-FL, in enteral formulas intended for patients with serious medical conditions is generally low and within the GRAS standard of reasonable certainty of no harm.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
Partially hydrolyzed guar gum (PHGG)				
Lampe et al., 1992	Prospective, randomized, placebo-controlled, double-blind, crossover study 11 healthy men	1. Self-selected diet 2. Enteral formula containing no added fiber (maltodextrin) 3. Enteral formula containing 15 g PHGG/day 4. Enteral formula containing 15 g soy polysaccharide	18 days with a 10 day - washout between each diet period	• 12 subjects completed the study; one man did not comply with the diet protocol and his data were excluded from the analyses. No other adverse events were reported. • Compared to the enteral diet with no fiber, fecal wet and dry weights, frequency, stool weight, fecal consistency, fecal moisture, and fecal pH were not statistically different, whereas mean transit time and fecal nitrogen were significantly increased in the PHGG-treated group. • Compared to the enteral diet with no fiber, fecal wet and dry weights, fecal nitrogen, frequency, stool weight, fecal consistency, and fecal pH were not statistically different, whereas mean transit time was significantly decreased and fecal moisture was significantly increased in the soy polysaccharide-treated group. • Colonic fluid acetate, propionate, butyrate and total short chain fatty acids were not significantly different between the PHGG- and no fiber-treated groups • The authors concluded that "despite significant differences in mean transit time, few differences in other parameters of bowel function were observed when healthy subjects consumed enteral formula diets containing 0 g of fiber and 15 g of total dietary fiber as modified guar and soy."
Meier et al., 1993	Randomized, placebo-controlled crossover study 12 healthy men	1. Standardized normal diet 2. Liquid formula diet 3. Liquid formula diet supplemented with PHGG; intake 42 g PHGG/day	7 days with a 7 day washout between each diet	• Significantly increased colonic but not orocecal transit time compared with either a self-selected diet or the enteral formula without fiber. • PHGG did not affect on stool consistency or frequency.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
Homann et al., 1994	Prospective, randomized, double-blind, placebo-controlled trial 100 hospital patients (30 receiving total enteral nutrition and 70 receiving enteral supplementation)	1. Standard diet 2. Standard diet with 20 g PHGG/L of formula; intake of TPN patients = 24 g PHGG/day; intake of enteral supplementation patients = 20 g PHGG/day	Total enteral nutrition was given for a minimum of 5 days	• Patient receiving either total or supplemental enteral nutrition had reduced incidence of diarrhea, but increased flatulence when receiving the standard diet with PHGG compared to those receiving the standard diet alone. • In the patients receiving total enteral nutrition, four patients on the standard total enteral diet, but no patients on the standard diet with PHGG discontinued due to diarrhea. • In the supplemental feeding groups, four patients receiving the standard diet vs. two receiving the standard diet with PHGG discontinued gastrointestinal side effects. • The authors, therefore, reported that: o The total number of patient with gastrointestinal side effects that resulted in discontinuation of the enteral feeding dropped from eight to two in the standard diet vs the standard diet with PHGG o The total number of GI-side effects was not different in the two groups (17 in each group).
Fussell et al., 1996 (Abstract)	Prospective, randomized, double-blind, placebo-controlled study 57 tube-fed adults in 5 diagnostic categories: abdominal surgery/ trauma, cerebral trauma, head/neck surgery, multiple fractures, and vascular surgery	1. Fiber free tube feeding formula 2. Fiber free tube feeding formula w/14 g PHGG/L of formula	5-14 days	• Forty-four patients completed the protocol. • There was no effect of the fiber on daily diarrhea, nor on albumin, transthyretin, or flatulence. • The PHGG was generally well tolerated.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
Peters and Davidson, 1996	Prospective, randomized, double-blind cross-over study 12 enterally fed patients with Type 1 diabetes	1. Formula containing 29% fat, 55% carbohydrate, and PHGG 2. Formula containing 40% fat, 44% carbohydrate, and PHGG 3. Formula containing 50% fat, 33% carbohydrate, and soy polysaccharide 4. Ensure (53% carbohydrate and no fiber	1 day with a week in between treatments	• The 2 formulas containing PHGG (concentration not specified) were not effective in attenuating the postprandial glucose response. • No adverse effects were reported.
Spapen et al., 2001	Prospective, randomized, double-blind, placebo-controlled study 25 ICU patients (13 M, 12 F; mean age = 68.5±13.1 years) with severe sepsis and septic shock fed enterally	1. Control formula 2. Formula containing 22 g PHGG/L of formula	At least 6 days	• The group receiving PHGG supplementation exhibited a significantly reduced frequency of diarrhea and a reduction in the number of days with diarrhea • PHGG supplementation had no significant effect on sepsis-related mortality (1 death in the test group, 4 in the control) or duration of stay in the intensive care unit. • The authors concluded: o "Fiber treatment was well-tolerated" o "Total enteral nutrition supplemented with soluble fiber is beneficial in reducing the incidence of diarrhea in tube-fed full-resuscitated and mechanically ventilated septic patients."
Homann et al., 2004	Prospective, randomized, double-blind, placebo-controlled trial 100 medical and surgical patients (50 patients per group); 30 patients received total enteral nutrition and 70 patients received 1000 ml/day supplemental enteral nutrition	1. Standard diet 2. Standard diet with 20 g PHGG/L of formula; intake of TPN patients = 24 g PHGG/day; intake of enteral supplementation patients = 20 g PHGG/day	Total enteral nutrition was given for a minimum of 5 days	• The PHGG-supplemented formula significantly reduced the number of patients with diarrhea (6 vs. 15 on the fiber-free formula) and the number of days patients suffered from diarrhea (10.2 vs. 40.6 days). • The number of patients experiencing GI side effects was the same in both groups (n = 17 per group), although flatulence was reported in more patients in the PHGG group. • Enteral nutrition was discontinued due to GI side effects in 4 patients on the control/standard diet, but no patients on the PHGG-supplemented diet.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
Rushdi et al., 2004	Prospective, randomized, double-blind, controlled study 30 IBS patients (11 M, 9 F; aged 28-73 years with mean age = 57/5±13/8 years) on enteral nutrition with 3 or more liquid stools/day	1. Standard fiber-free feed 2. Enteral feed enriched with 222 g PHGG/L (22 to 37 g PHGG/day)	4 days	• 20 patients completed the protocol (n=10/group); the ten patients that did not complete the protocol because they switched to parenteral nutrition or oral diet, death, or leaving the ICU before completing the study. • Supplementation with PHGG significantly reduced the number of liquid stools. • There were no differences in the incidence or severity of gastrointestinal symptoms between the two groups. • The authors discussed tolerance issues extensively: "Throughout the course of this clinical trial, in the fiber- enriched feed group, only two patients complained of flatulence (20%). On the other hand, in the control group, four patients complained of flatulence (40%), two patients got vomiting (20%) and one case of constipation (10%) was reported. However, no statistical significance was found between both groups as regards incidence or severity of gastrointestinal symptoms. None of these symptoms was severe enough to necessitate therapeutic intervention."
Galactomannan				
Nakao et al., 2002	Open-label study 20 elderly bed-ridden males and females (10 M, 10 F, mean age = 79.3±5.1 years) receiving enteral feeding	A semi-digested formula containing galactomannan 7 g galactomannan/day during the first week; the dose was increased 7 g/day each week until they received 28 g galactomannan/ day for the fourth week	4 weeks	• No adverse effects were reported. • Serum diamine oxidase activity significantly increased following the treatment with the semidigested formula containing galactomannan. • The water content of the feces decreased, and the frequency of normal stools increased with the semidigested formula containing galactomannan. • The frequency of bowel movements, the number of aerobic bacteria, and the pH of feces decreased, while fecal SCFA, especially acetic and propionic acids, increased with the semidigested formula containing galactomannan. • All effects reversed after termination of the galactomannan supplementation. • There was no change in counts of total bacteria or anaerobes and no change in body weight, total serum protein, prealbumin, transferrin, retinol-binding protein, total cholesterol, triacylglycerol, iron, copper, or zinc.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
Fructooligosaccharides				
Karakan et al., 2007	Randomized, double-blind, placebo-controlled study 30 patients aged 46.1±14.0 years with severe acute pancreatitis requiring stoppage of oral feeding for 48 hr	1. Diet 2. Diet containing 0.7 g/soluble fiber and 0.8g/100 g insoluble fiber (24 g/day)	2 days	• Both enteral feeding solutions were well tolerated with no reported adverse effects. • The median duration of enteral feeding and the hospital stay was significantly shorter in the group receiving the fiber-containing diet. • The fiber-containing diet also significantly improved the pancreatitis severity scores. • The authors concluded that nasojejunal EN with fiber supplementation in severe AP improves hospital stay, duration of nutrition therapy, acute phase response and overall complications compared to standard EN therapy.
Khoshoo et al., 2010	Randomized, double-blind crossover study 14 children aged 1-15 years receiving 75-100% of calories via feeding tube and were candidates for receiving a peptide-based enteral formula based on documented gastrointestinal dysfunction	1. Formula 2. Formula with 3.5 g FOS/L (approximately 3.5 g FOS/ day)	2 weeks with a 5-day washout period between treatment periods	• There were nine patients with neurological disorders; 3 patients with inflammatory bowel disease; and 2 patients with short bowel syndrome • There were no withdrawals. • There was no significant difference in the daily number of bowel movements between children receiving either the fiber or control formulas when evaluating the three diagnoses groups combined or the short bowel syndrome group alone. • The children with neurological impairments had more frequent bowel movements when fed the control formula than when fed fiber formula whereas the inflammatory bowel disease group had more daily bowel movements when fed the fiber-containing formula • Stools were in the "mushy" category when the participants consumed the fiber containing formula • Children with neurological impairment had a significantly lower proportion of stools ($P<0.05$) characterized as hard nuts and a significantly lower proportion of stools. • In the inflammatory bowel disease group, stool frequency was higher with the fiber formula, but there was no change in consistency. • There was no difference in the occurrence of vomiting between the two treatments in any of the groups • The nine children with a neurological disorder, the mean grade of flatulence/gas was significantly less ($P<0.05$) when participants consumed the fiber formula whereas there was no difference in flatulence in the other groups.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
				• There were no differences in abdominal pain or weight gain among the different groups. • The authors concluded, "This study showed that a peptide-based formula containing fiber was as well-tolerated as a fiber-free formula in a small population of children with gastrointestinal impairments."
Garleb et al., 1996	Randomized, double-blind, controlled study 27 healthy male college students (n=9/treatment group)	1. Formula 2. Formula with 5 g scFOS/L (approx. 15 g scFOS/day) 3. Formula with 10 g scFOS/L (approx. 30 g scFOS/day)	14 days	• One subject dropped out of the study after one day due to intolerance to the liquid product. The subject was replaced with an alternate. • There were no differences in body weight or deviations from the normal range of blood chemistry values among the three treatment groups. • Although there were no differences in propionate or butyrate, fecal pH, or fecal percent dry matter, fecal acetate, isobutyrate, and isovalerate concentrations were higher among students ingesting scFOS. • Consumption of scFOS also increased fecal bifidobacteria. • Complaints of nausea, cramping, distension, vomiting, diarrhea, and regurgitation were similar across all groups and were present on fewer than 5% of participant-days. • Flatus was reported more frequently by those consuming 30 g scFOS/day, but most complaints occurred during the first 4 days. • The authors concluded that "these results indicate that [scFOS] does not compromise serum chemistry profiles, is well tolerated particularly at an intake of 15 g/d and would serve as a bifidogenic factor when incorporated into a liquid enteral product."
Simakachorn et al., 2011	Randomized, double-blind, placebo-controlled study 94 critically ill children age 1-3 years under mechanical ventilation and enteral feeding (n=47/groups)	1. Control formula 2. Test formula with 2.6 g/L of oligo-fructose/inulin and 2.8 g/L of acacia gum in combination with 2 strains of live microorganisms	7 days of enteral feeding followed by 14 days of oral feeding	• 6 children withdrew from the test formula group; 8 children withdrew from the control formula group. One child withdrew consent in the test formula group, 5 children withdrew consent in the control formula group. • One child was lost to follow-up in the test formula group (moved to another hospital) and one child was lost to follow-up in the control formula group (no reason given). Four children discontinued the intervention in the test formula group due to death whereas two children discontinued the intervention in the control formula group due to death. • There were no significant differences in adverse events between the two groups and no reported secondary infections during the ICU stay. • Abdominal distension, vomiting, and stool frequency were also unaffected by the fiber.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
				• The authors concluded that the experimental enteral formula is safe and well tolerated by children in intensive care receiving enteral nutrition.
Majid et al., 2014	Randomized, double-blind, placebo-controlled study 47 adults in the intensive care unit	1. Control formula containing soy polysaccharides, resistant starch, Arabic gum, cellulose, inulin, and oligofructose (0.7 g/100 ml soluble fiber and 0.8 g/100 ml insoluble fiber, equivalent to 6.75 g/day)); n=23 2. Formula containing soy polysaccharides, resistant starch, Arabic gum, cellulose, inulin, and oligofructose (0.7 g/100 ml soluble fiber and 0.8 g/100 ml insoluble fiber; equivalent to 6.75 g/day) with and additional 7 g oligofructose/inulin; n=24	A minimum of 3 days	• 12 patients discontinued the study before the intervention (7 in the placebo group and 5 in the oligofructose/inulin group) • 6 patients discontinued the intervention in the control formula group (1 patient transferred to an oral diet and five transferred to palliative care) vs 7 patients discontinued in the oligofructose/inulin group (5 transferred to palliative care and 2 were discharged to another hospital) • There was no significant difference in short-chain fatty acid concentrations at baseline or follow-up between the two groups. • Fecal pH was similar in the two groups at baseline and at follow-up. • There were no significant differences in fecal frequency or the daily fecal score between the two groups. • There was no difference between the two groups in the mean number of days of diarrhea or in the number of patients experiencing diarrhea on either one or two or more consecutive days.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
Galactooligosaccharides or GOS/FOS				
Modi et al., 2010	Prospective, randomized, double-blind, placebo-controlled, multi-center study 160 preterm infants (gestational age <33 weeks) receiving enteral feeding	1. Standard formula 2. Test formula with 8 g/L of scGOS/lc FOS in a 9:1 ratio	~8 weeks or until discharge	• 83 infants received the standard formula; 77 infants received the test formula containing GOS/FOS. The parents of two and four infants withdrew consent in the standard and test formula groups, respectively. One infant in the standard formula group died before reaching the primary outcome and two infants in the test formula group died before reaching the primary outcome. One infant in the standard formula treated group was discharged before reaching the primary outcome. • Six adverse events were reported by one infant, five of which were not considered related to the trial. • There were three cases of necrotizing enterocolitis (one in the standard formula group vs 2 in the test formula group). • Nineteen infants develop at least one episode of a blood stream infection (10 in the standard formula group vs 9 in the test formula group. • There was no overall difference in tolerance between control and test formula, but the addition of scGOS/lc FOS to formula improved tolerance for the most immature infants. There were no differences in gains in weight, length, or head circumference; in stooling frequency, stool characteristics, or fecal microbiota; or in GI signs or water balance (based on concentrations of serum sodium and creatinine). • The authors concluded that scGOS/lc FOS supplementation is safe.
Akatsu et al., 2016	Prospective, randomized, double-blind, placebo-controlled study 36 elderly individuals	1. Oral feeding (n=13) 2. Enteral formula (n=11) 3. Enteral formula w/ GOS and (BGS; 2-amino-3-carboxy-1,4-naphtho-quinone) (n=12) Products were delivered via percutaneous endoscopic gastrostomy	10 weeks	• No adverse effects were reported.

Table 18. Clinical Studies of Non-digestible Carbohydrates Administered Via Enteral Feeding[1]

Citation	Study Design	Treatments	Duration	Safety-Related Findings
Armanian et al., 2016	Prospective, randomized, double-blind, placebo-controlled study 25 hyper-bilirubinemic preterm neonates who had reached 30 ml/kg bw/day enteral feeding volume	1. Distilled water 2. A supplement containing scGOS/lc FOS in a 9:1 ratio *The supplement was initially administered by 0.5 g/kg/day and then increased to 1 g/kg/day and 1.5 g/kg/day	1 week	• No adverse effects were reported. • Stool frequency was significantly increased in the scGOS/lc FOS-treated group. • The authors concluded that oligosaccharides increase stool frequency, improve feeding tolerance and reduce bilirubin level in preterm neonates and therefore can be efficacious for the management of neonatal hyperbilirubinemia.
Van den Berg et al., 2015	Prospective, randomized, double-blind, placebo-controlled study to determined the effect of combined short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS) and pectin-derived acidic oligosaccharides (pAOS) on antibody concentrations after pneumococcal conjugate vaccination in very preterm infants. 113 infants with a gestational age of <32 weeks or birth-weight <1500 g	1. Placebo/maltodextrin (n=58) 2. scGOS/lc FOS/ pectin-derived acidic oligo-saccharides(pAOS) (n=55)	4 weeks	• Nine infants died in the placebo-treated group whereas six infants died in the scGOS/lc FOS/pAOS-treated group. • Adverse events were not reported. • The authors concluded "Short-term supplementation of scGOS/lcFOS/pAOS during day 3–30 of life decreased the pneumococcal vaccine antibody response after the primary series of PCV7 at 5 months in preterm infants to levels which are similar in term infants from a Dutch population study. However, after the booster vaccination at 12 months, this effect of the scGOS/lcFOS/pAOS on the PCV response had disappeared."

[1]Incorporated by reference from the amendment to GRN 897.

3. Clinical Studies with Other Non-digestible Carbohydrates and Oral Electrolyte Solutions

a. Background

Oral electrolyte solutions (OESs) are liquid products that facilitate rapid and effective rehydration. OESs contain, at a minimum, a digestible carbohydrate such as dextrose and sodium in water to facilitate water absorption from the lumen of the gastrointestinal tract. Specifically, dextrose absorption facilitates sodium ion absorption, which thereby raises the concentration of sodium ions in the blood stream, pulling water from the lumen of the gastrointestinal tract into the blood stream. Importantly, this is all accomplished through a balance between the amount of carbohydrate and the electrolytes in the OES. Additionally, although sodium absorption improves as the dextrose concentration of the oral fluid is increased up to about 2.5% w/w, higher concentrations of dextrose can increase the osmotic load in the gut, pulling water out of the blood stream, further exacerbating dehydration. Simple sugars such as dextrose and fructose have also been shown to be more effective than larger, more complex carbohydrates in facilitating electrolyte absorption and many oligosaccharides are not stable in acidic mediums such as OESs. As a result, conventional OESs generally do not include oligosaccharides or polysaccharides (Patent 10,695,358, date issued June 30, 2020, Abbott Laboratories).

Importantly, non-digestible carbohydrates, such as 2'-FL, GOS, FOS and LNnT stimulate the growth or activity, or both, of Bifidobacterium in the gastrointestinal tract (reviewed in Gibson and Roberfroid, 1995). Non-digestible carbohydrates are also fermented by the colonic bacteria to short-chain fatty acids (SCFA), which are rapidly absorbed in the colon and further promote fluid and sodium absorption (reviewed in Binder et al., 2014). Thus, OESs supplemented with non-digestible carbohydrates, such as 2'-FL, may facilitate rehydration, as well as maintenance of the microbiota.

b. Use of Non-Digestible Carbohydrates in Acute Diarrhea and As an Ingredient in Oral Electrolyte Solutions

The safety and tolerance of numerous non-absorbable carbohydrates (GOS, FOS, xylooligosaccharides (XOS)) have been extensively reviewed and been the subject of numerous GRAS Notices (GRNs 44, 172, 233, 236, 246, 285, 286, 334, 343, 370, 458, 484, 495, 518, 537, 569, 605, 620, 623, 671, 674, 717, 721, 729, 779, 797, 816, 818, 896); human milk oligosaccharides have also been extensively reviewed and the subjects of numerous GRAS Notices (2'-FL: GRNs 546, 571, 650,735, 749, 815, 852, 859, 897; 3-FL: GRN 925; 3'-SL and 6'-SL: GRNs 766, 880, 881, 921, 922; LNT: GRN 923; LNnT: GRNs 919, 895).

During diarrhea, pathogenic bacteria may either grow and colonize the gastrointestinal (GI) tract and then invade the host tissues or, alternatively, they may secrete toxins which may disrupt the function of the intestinal mucosa, causing nausea, vomiting, and diarrhea. Oli et al., (1998) showed that in a pig model, adding fructo-oligosaccharides (FOS) to an OES accelerated the recovery of lactobacilli and reduced bacterial counts of Enterobacteriaceae. Brunser et al. (2006) studied the effect of FOS on the intestinal microbiota during treatment with amoxicillin and reported an increase in bifidobacteria in patients receiving FOS after seven days of antibiotic treatment compared to a control group. These authors reported that the effect of FOS on the occurrence of antibiotic-related diarrhea episodes was not significant. Vaisman et al. (2010) investigated the effect of a mixture of long-chain FOS, GOS, and acidic oligosaccharides on the number and consistency of stools and on immune system biomarkers in 104 supplemented and non-supplemented subjects (aged 9–24 months) with acute diarrhea. No treatment-related adverse effects were reported. Additionally, studies of OESs supplemented with non-digestible carbohydrates and/or sources of non-digestible carbohydrates, such as guar gum, FOS, XOS, and high amylose maize starch, indicate that non-digestible carbohydrates do not exacerbate acute diarrhea (Table 19; Alam et al., 2015; Passariello et al., 2011; Vandenplas et al., 2011; Raghupathy et al., 2006; Hoekstra et al., 2004; Alam et al., 2000). Therefore, based on the weight of the evidence, adverse effects resulting from the addition of 2'-FL to OESs are not expected.

c. Lack of Impact of 2'-FL on Osmolarity

The World Health Organization (WHO) current standard OES osmolarity is 245 mOsm/L; Pedialyte® from Abbott is 250 mOsm/L (Ofei et al., 2019). Despite common perceptions that sport drinks can be used for dehydration, liquid products such as sports beverages and juices are hyperosmolar (330–730 mOsm/L) and inappropriate as rehydration solutions for diarrhea and dehydration because they increase fluid losses and worsen the diarrheal disease. It is critical that the addition of any ingredient to an OES not impact the osmolarity. The addition of 1.2 g/L of 2'-FL to OES, such as Pedialyte®, is calculated on the basis of molar weight to add 2.5 mOsm/L, thus it will not impact the osmolarity of the solution.

Table 19. Studies of Oral Electrolyte Solutions (OES) with Added Non-digestible Carbohydrate

Reference	Trial Design	Test Article	Results
Alam et al., 2015	Randomized, double-blind placebo controlled clinical trial of 126 malnourished children (male and female) (weight for length/weight for age <3 Z-score with or without pedal edema), aged 6-36 months with acute diarrhea	• Group 1: Standard hypotonic oral rehydration solution (ORS) • Group 2: Standard hypotonic ORS with 15 g/L partially hydrolyzed guar gum	• The mean duration of diarrhea was significantly shorter in children in Group 2 compared to Group 1. • Adverse events/tolerance related to test article not reported by authors.
Passariello et al., 2011	Single-blind, prospective, controlled trial including children (age range, 3-36 months) with acute diarrhea	• Group 1: Standard hypotonic oral rehydration solution (ORS) • Group 2: hypotonic ORS with zinc, 0.35 g/L fructooligosaccharides, and 0.35 g/L xylooligosaccharides	• Resolution of diarrhea at 72 hours, number of daily outputs at 24, 48, and 72 hours was statistically significantly improved in Group 2 compared to Group 1. • Total ORS intake in the first 24 hours of rehydration therapy was statistically significantly lower in Group 1 than Group 2. • No adverse events related to the use of the ORS were observed in the study groups.
Vandenplas et al., 2011	Randomized, prospective, double-blind placebo-controlled trial in children between 3 and 186 months (males and females) with acute diarrhea	• Group 1: Standard hypotonic oral rehydration solution (ORS) • Group 2: Standard hypotonic ORS with a symbiotic blend (*Streptoccoccus thermophilus*, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, *Bifidobacterium lactis*, *Bifidobacterium infantis*, fructooligosaccharides).	• Children in Group 2 had significantly reduced duration of diarrhea compared with Group 1. • Adverse events/tolerance related to test article not reported by authors.
Raghupathy et al., 2006	Randomized, double-blind, placebo-controlled study including boys aged 6 months to 3 years with acute diarrhea with clinically detectable dehydration	• Group 1: Standard hypotonic oral rehydration solution (ORS) (311 mOsm/kg) • Group 2: Standard hypotonic ORS with 50 g/L high-amylose maize starch	• Statistically significant shortened duration of diarrhea in Group 2 compared to Group 1. • Before the start of this study high-amylose maize starch, ORS was administered orally to 6 children with acute diarrhea and found to be well tolerated. It did not induce vomiting or significantly increase in diarrhea.

Table 19. Studies of Oral Electrolyte Solutions (OES) with Added Non-digestible Carbohydrate			
Reference	**Trial Design**	**Test Article**	**Results**
Hoekstra et al., 2004	Randomized, double-blind, placebo-controlled multicenter study including boys aged 1 to 36 months with acute diarrhea	• Group 1: Standard hypotonic oral rehydration solution (ORS) • Group 2: Standard hypotonic ORS with a mixture of non-digestible carbohydrates (soy polysaccharide 25%, alpha-cellulose 9%, gum arabic 19%, fructooligosaccharides 18.5%, inulin 21.5%, resistant starch 7%)	• No significant differences in mean 48 hours stool volume or duration of diarrhea in Group 2 compared to Group 1. • No significant adverse effects, as compared to ORS with placebo, were noted.
Alam et al., 2000	Double-blind, randomized, placebo controlled clinical trial of 150 male children aged 4 to 18 months who had acute diarrhea	• Group 1: Standard hypotonic oral rehydration solution (ORS) • Group 2: Standard hypotonic ORS with 15 g/L partially hydrolyzed guar gum	• Children in Group 2 had significantly reduced duration of diarrhea compared with Group 1. • Adverse events/tolerance related to test article not reported by authors.

G. ALLERGENICITY

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

H. REGULATORY APPROVALS AROUND THE WORLD

In the United States, 2'-FLis GRAS for use in non-exempt infant formulas at levels up to 2.4 g/L, exempt infants formulas at levels up to 2.0 g/L, selected conventional foods and beverages and enteral tube feeding formulas at levels ranging from 0.28 to 1.2 g/serving (GRN 546, 2015; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019). Eleven 2'-FL GRAS Notifications have been filed with FDA, seven of which received "no questions" letters. FDA's review of two of the 2'-FL GRAS Notifications was ceased due to major deficiencies and one was due to questions regarding support for an intended use level of 3.64 g/L. A mixture of 2'-FL and difucosyllactose is also GRAS for use in infant formula, toddler formula, drinks for young children and selected conventional foods and beverages (GRN 815, 2019).

Outside the United States, 2'-FL is a Novel Food in the European Union and approved for use in infant formula and selected foods alone or in combination with lacto-*N*-*neo*tetraose at levels up to 1.2 g/L and 200 g/kg, respectively. It is also a Novel Food in Canada and authorized in Malaysia, Taiwan, Singapore, Israel, and the Philippines. In Australia and New Zealand, 2'-FL and LNnT are currently the subjects of a Novel Food application and the Food Standards of Australia and New Zealand has concluded that there are no public health and safety concerns associated with the addition of 2'-FL alone or in combination with LNnT to infant formula products and FSFYC at the requested levels, or at higher estimated levels of dietary intakes based on 2.4 g/L 2'-FL (Food Standards Australia New Zealand, 2018).

VII. SUPPORTING DATA AND INFORMATION

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Zhou, Y., Kruger, C., Ravi, G.S., Kumar, D.P.S., Vijayasarathi, S.K., Lavingia, M., Chen, X., and Ambriz, P. (2017). Safety evaluation of galacto-oligosaccharides: subchronic oral toxicity study in Sprague-Dawley rats. Toxicology Research and Application *1*, 1–12.

B. EXPERT PANEL STATEMENT

We, the members of the Expert Panel, qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food, have performed a comprehensive and critical review of available information and data on the safety and Generally Recognized As Safe (GRAS) status of 2'-Fucosyllactose (2'-FL) in toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas has been shown to be safe and GRAS, using scientific procedures, under the Federal Food, Drug, and Cosmetic Act (FFDCA), as described under 21 CFR §170.30(b). The safety of the intake of 2'-FL in toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas has been determined to be GRAS by demonstrating that the safety of this level of intake is generally recognized by experts qualified by both scientific training and experience to evaluate the safety of substances directly added to food and is based on generally available and accepted information.

The use of 2'-FL as an ingredient for the intended use in toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas has been determined to be safe through scientific procedures set forth under 21 CFR §170.30(b) based on the following:

1. The subject of this GRAS Determination is a spray-dried, powdered food ingredient that contains not less than 90 % 2'-FL dry weight. The remaining components include carbohydrate by-products, ash, and moisture

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

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

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

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

a. The subject of this GRAS Determination is manufactured using a genetically engineered strain of *Escherichia coli* BL21(DE3) by Chr. Hansen A/S in Food Safety System Certification (FSSC) 22000-, ISO 9001:2015-, GMP-, and International Featured Standards Food 6.1-compliant facilities. Chr. Hansen A/S is a Food Facility registered with FDA.

b. The genetically engineered strain of *Escherichia coli* BL21(DE3) used by Chr. Hansen A/S is non-toxigenic, not capable of DNA transfer to other organisms, and has the same virulence profile as *E. coli* BL21(DE3).

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

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

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

f. Use of the subject of this GRAS determination in the intended selected conventional foods and enteral tube feeding formulas results in mean and 90th percentile estimated daily intakes (EDIs) of 2.16 and 5.26 g/day (0.032 and 0.078 g/kg bw/day) for consumers not less than 2 years-old.

g. Use of the subject of this GRAS determination in selected conventional foods and enteral tube feeding formulas results in mean and 90th percentile cumulative estimated daily intakes (EDIs) of 2.5 and 5.16 g/day (0.037 and 0.077 g/kg bw/day) for consumers not less than 2 years-old.

h. The use of the subject of this GRAS determination in oral electrolyte solutions results in an estimated daily intake of 1.2-2.4 g of 2'-FL (equivalent to 88.9-177.8 mg of 2'-FL/kg bw/day assuming a 13.5 kg toddler and 17.1-34.3 mg of 2'-FL/kg bw/day assuming a 70 kg adult). Because OESs are intended for short term use, intake of 2'-FL from OESs will not impact the cumulative 2'-FL intake resulting from the use of 2'-FL in select conventional foods and enteral tube feeding formulas.

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

4. The safety of exposure to Chr. Hansen A/S's 2'-FL ingredient at its intended use level is supported by:

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

    b. Published tolerance studies in neonatal piglets showing that the ingestion of up to 3.92 g/L of the subject of this GRAS determination alone or in the presence of other HMOs was well-tolerated and supported normal growth in neonatal piglets;

    c. Clinical data showing the ingestion of HMOs are well tolerated in infants up to 1 g/day and adults up to 20 g/day;

    d. Clinical data showing that the use of other non-digestible carbohydrates in infants, adults, enteral tube feeding products and oral electrolyte solutions is well tolerated up to 63 g/day;

    e. The GRAS status of the subject of this Determination for use in non-exempt term infant formula (GRN 571);

    f. The GRAS status of other 2'-FL products for use in non-exempt term infant formula, selected conventional foods and enteral tube feeding formulas (GRN 546, 2014; GRN 571, 2015; GRN 650, 2016; GRN 735, 2018; GRN 749, 2018; GRN 852, 2019; GRN 897, 2020).

Therefore, 2'-FL is safe and GRAS at the proposed level of addition to the intended toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas. 2'-Fucosyllactose is, therefore, excluded from the definition of a food additive, and may be used in the U.S. without the promulgation of a food additive regulation by the FDA under 21 CFR.

Peter Pressman, MD, MS, FACN, Signature: [redacted]
GRAS Expert Panel Member
Medicine Public Health & Nutrition Date: May 18, 2021
The Daedalus Foundation

A. Wallace Hayes, PhD, DABT, FATS, ERT Signature: [redacted]
GRAS Expert Panel Member
Harvard School of Public Health Date: May 18, 2021

Thomas E. Sox, PhD, JD Signature: [redacted]
GRAS Expert Panel Member
Principal, Pondview Consulting LLC Date: May 18, 2021

Claire Kruger, PhD, DABT Signature: [redacted]
Scientific Advisor to the Panel
Date: May 18, 2021

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
001014	June 8, 2021
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

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

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

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

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

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Kate Urbain	Head of Regulatory Affairs North America

Organization *(if applicable)*: Chr. Hansen A/S

Mailing Address *(number and street)*: 9015 W Maple St.

City	State or Province	Zip Code/Postal Code	Country
West Allis	Wisconsin	53214	United States of America

Telephone Number	Fax Number	E-Mail Address
414-607-5819		USKAUR@chr-hansen.com

1b. Agent or Attorney *(if applicable)*

Name of Contact Person	Position or Title
Dietrich B. Conze	Managing Partner

Organization *(if applicable)*: Spherix Consulting Group, Inc.

Mailing Address *(number and street)*: 751 Rockville Pike, Unit 30-B

City	State or Province	Zip Code/Postal Code	Country
Rockville	Maryland	20852	United States of America

Telephone Number	Fax Number	E-Mail Address
240-367-6089		dconze@spherixgroup.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

2'-Fucosyllactose (2'-FL)

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

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

3. For paper submissions only:

Number of volumes _____

Total number of pages _____

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

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

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

☒ a) GRAS Notice No. GRN 571

☐ b) GRAS Affirmation Petition No. GRP _____

☐ c) Food Additive Petition No. FAP _____

☐ d) Food Master File No. FMF _____

☒ e) Other or Additional *(describe or enter information as above)* GRN 546, 650, 659, 735, 749, 766, 815, 852, 880, 897, 919, 921, 922

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

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

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

☐ No

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

☐ Yes, a redacted copy of the complete submission

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

☐ No

SECTION D – INTENDED USE

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

Chr. Hansen A/S intends to use 2'-FL as an ingredient in toddler formulas, foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas.

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

(Check one)

☐ Yes ☒ No

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

(Check one)

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

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

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

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

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

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

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

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

Other Information

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

☐ Yes ☒ No

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

☐ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Chr. Hansen A/S
(name of notifier)

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

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

2. Chr. Hansen A/S agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.
(name of notifier)

9015 W Maple St, West Allis, WI 53214
(address of notifier or other location)

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

3. Signature of Responsible Official, Agent, or Attorney	Printed Name and Title	Date (mm/dd/yyyy)
Dietrich B. Conze, PhD Digitally signed by Dietrich B. Conze, PhD Date: 2021.06.04 10:17:17 -04'00'	Dietrich B. Conze, PhD, Managing Partner	06/04/2021

SECTION G – LIST OF ATTACHMENTS

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

Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Chr Hansen 2	issio
	Reference	ission

From: kbrailer@spherixgroup.com
To: Hice, Stephanie
Cc: "Dietrich Conze"; ckruger@spherixgroup.com
Subject: [EXTERNAL] GRN 001014 - Questions for Notifier
Date: Friday, January 21, 2022 4:32:34 PM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.png
image006.png
Chr Hansen Response to FDA on GRN1014 1-21-22.pdf

Dear Dr. Hice,

Attached please find our response to your request for additional information on GRN 001014. Please confirm receipt and let us know if you need anything else.

Best regards,

Kathy Brailer
Director of Administrative Services
Spherix Consulting Group, Inc.
751 Rockville Pike, Unit 30-B
Rockville, MD 20852
+1-301-557-0375
kbrailer@spherixgroup.com
www.spherixgroup.com

From: Hice, Stephanie Stephanie.Hice@fda.hhs.gov
Sent: Monday, December 20, 2021 2:55 PM
To: Dietrich Conze dconze@spherixgroup.com
Cc: Claire Kruger ckruger@spherixgroup.com; Kathy Brailer kbrailer@spherixgroup.com
Subject: RE: [EXTERNAL] Re: GRN 001014 - Questions for Notifier

Dear Dr. Conze,

Thank you for reaching out. Yes, January 21, 2022 would be fine.

Wishing you a happy new year!

Sincerely,

Stephanie Hice

Stephanie Hice, Ph.D. (they/them/their)
Regulatory Review Scientist & Microbiology Reviewer

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
stephanie.hice@fda.hhs.gov

Pronouns: They-Them-Their (what is this?)

From: Dietrich Conze <dconze@spherixgroup.com>
Sent: Monday, December 20, 2021 2:47 PM
To: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Cc: Claire Kruger <ckruger@spherixgroup.com>; Kathy Brailer <kbrailer@spherixgroup.com>
Subject: [EXTERNAL] Re: GRN 001014 - Questions for Notifier

Hi Stephanie,
Thank you for your questions. To accommodate the upcoming holidays and vacation plans, we would like extend the response time for sending you our responses to January 21, if possible.
Regards.
Dietz

Dietrich Conze, PhD
Managing Partner
Spherix Consulting Group
751 Rockville Pike, Unit 30-B
Rockville, MD 20852

Tel: 240-367-6089
Fax: 301-230-2188
dconze@spherixgroup.com

On Dec 20, 2021, at 10:28 AM, Hice, Stephanie <Stephanie.Hice@fda.hhs.gov> wrote:

Dear Dr. Conze,

During our review of GRAS Notice No. 001014, we noted questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your response.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

Stephanie Hice, Ph.D. (they/them/their)
Regulatory Review Scientist & Microbiology Reviewer

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
stephanie.hice@fda.hhs.gov

Pronouns: They-Them-Their (what is this?)

<image013.png>
<image014.png> <image015.png> <image016.png> <image017.png> <image018.png>

<2021-12-20 GRN 1014 - Questions for Notifier.pdf>

Spherix Consulting Group

January 21, 2022

Stephanie Hice, Ph.D.
Regulatory Review Scientist & Microbiology Reviewer
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
5001 Campus Drive, HFS-225
College Park, MD 20740

RE: Questions Regarding GRN 001014

Dear Dr. Hice:

Below are our responses to your requests for additional information regarding GRN 001014 as stated in your email on December 20, 2021. Your requests are in italicized text and our responses are in plain text below. Also, while preparing our answers to your questions, we found an error in Table 7 of the GRAS Notice. Specifically, the use level of 6 g/L that is listed as GRAS in enteral and tube feeding formula is wrong and should be 20 g/L, as stated in GRN 000897.

1. *The notifier states that 2'-fucosyllactose (2'-FL) is intended for use in milk-based meal replacement beverages for children (Table 1, page 1). Please specify if this implies cow and/or soy milk or other milk analogs.*

   The intended use in milk-based meal replacement beverages encompasses meal replacement beverages for children, such as PediaSure or Boost Kid Essentials. These products often contain a combination of milk and soy protein ingredients, in addition to other macro- and micro-nutrients. These products are covered in previous GRAS notifications (GRN 000897; GRN 000852; GRN 000735; GRN 000650), referenced as meal-replacement beverages.

2. *In the intended uses section please specify the age ranges for "children" and "young children". (Table 1, page 1). Please also specify the uses attributed to the different age groups listed in detail in the dietary exposure section (Tables 8 and 9, page 25).*

   The intended uses listed in Table 1 and Table 7 that specify "for infants and young children" and "for children" refer to products listed as "baby food" or "baby juice" in the NHANES database. The food codes for these products are 56210000, 578XXXXX, 1148XXXX, 67250100, 67250150, 6740XXXX, 67413700, 67430500, 13310000, 13311000, 13312000, 5380XXXX, 543XXXXX, 54408100, 57830100, 67100110, 6740XXXX, 6741XXXX, 67430000, and 672XXXXX, where X denotes any digit from 0-9.

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Rockville, MD 20852
301-557-0375; info@spherixgroup.com
http://spherixgroup.html

Importantly, the NHANES database does not specify an age range for these products. Additionally, the estimated daily intakes provided in Tables 8 and 9 were determined by querying the NHANES database with food codes that approximate the intended uses. The unique users of the food codes identified in the query were then binned according to age group (noted in Table 8 and 9), and the 2'-FL intakes for each unique user and each food code was then calculated by multiplying amount of the food consumed for each food code by the 2'-FL inclusion rate. The 2'-FL intakes for each unique user and each food code within each age group were then compiled to obtain the distribution of total 2'-FL intakes within each age group. Because the data in the NHANES database relies on real-world use of the foods associated with each food code, the method provides an accurate representation of the estimated daily intake of the ingredient. The method also prevents bias that may occur if specific uses and/or food codes were assigned to specific age group. It is therefore not possible to specify the uses attributed to the different age groups listed in detail in the dietary exposure section.

3. *Please provide a representative chromatogram for each monosaccharide for the sugars that are analyzed by HPAEC-PAD method (Table 2, page 10).*

A representative chromatogram for 2'-FL (Batch No. 26120020) analyzed by HPAEC-PAD, along with an overlay of the chromatograms for commercially purchased standards for residual carbohydrates is provided in Figure 1.

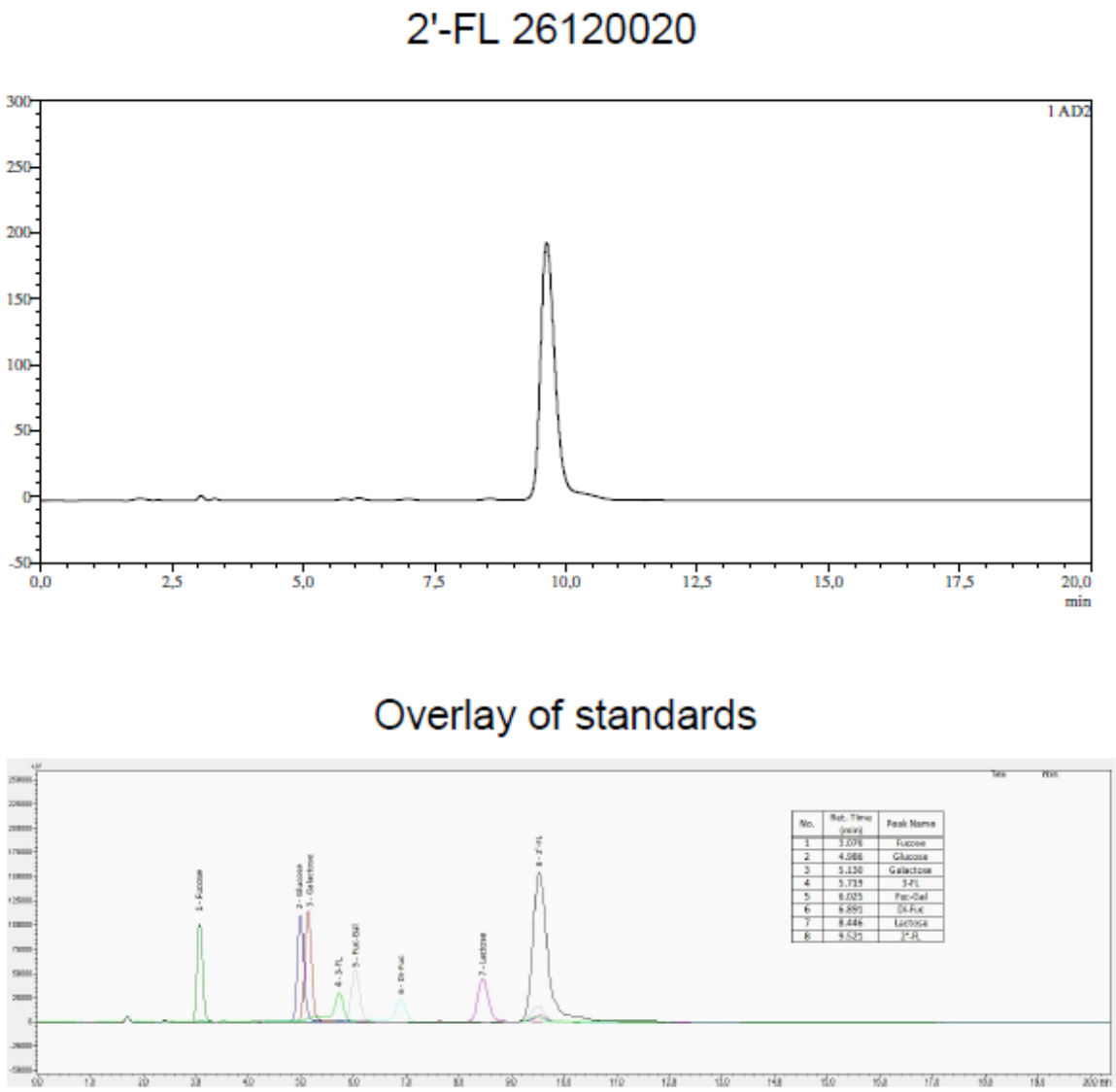


Figure 1. Representative HPAEC-PAD Chromatogram for 2'-FL (Batch No. 26120020), Along with an Overlay of the Chromatograms for the Carbohydrate Standards

Spherix Consulting Group, Inc.
751 Rockville Pike, Unit 30B, Rockville, MD 20852
301-557-0375; info@spherixgroup.com

4. *In Table 7, the notifier cites that the use level of 2′-FL in oral electrolyte solutions (OES) is GRAS at a use level of 1.2 g/L. Please clarify the GRAS notice, if submitted to FDA, that the notifier refers to in this statement.*

   The use of 2′-FL in OES has not been determined GRAS previously. The inclusion of 1.2 g/L in this row and column of Table 7, entitled "Use Levels That are GRAS (g/kg or g/L), is a typographical error and should be listed as "-".

5. *Please clarify if 2′-FL will be added to OES as stated on page 77 of the notice, and is not a stand-alone use. Also, please provide the formulation of the OES containing 2′-FL that is intended as the article of commerce.*

   There is a typographical error in first sentence of the first paragraph of Chapter III, Section D.1, entitled "Estimated Daily Intake of 2′-FL from Oral Electrolyte Solutions". 2′-FL is intended to be added to OES to prevent dehydration caused by vomiting, diarrhea, or heat exhaustion. This is a stand-alone use and does not include products that prevent dehydration caused by exercise or travel. Importantly, the intended uses of 2′-FL in drinks intended for rehydration after exercise or travel are covered by the category "sports, isotonic, and energy drinks" in Table 7, which was included in the estimated daily intake assessments provided in Tables 8 and 9 of the Notice.

   Regarding the formulation of the OES containing 2′-FL, the formulation of the OES that is intended to be used to prevent dehydration caused by vomiting, diarrhea, or heat exhaustion has an osmolarity of 250 mOsm/L, which approximates the osmolarity recommended by the World Health Organization for oral rehydration solutions (245 mOsm/L). OESs are composed of a combination of water, dextrose, and minerals (electrolytes), and in some formulations, additional ingredients such as galactooligosaccharides, flavorings, sweeteners, and color additives. Additionally, as stated in Chapter IV. Section F.3.c (pg. 77), the addition of 1.2 g/L of 2′-FL to OES will add 2.5 mOsm/L to the OES, resulting in an osmolarity of 252.5 mOsm/L, and will not significantly increase the osmolarity of the resulting OESs formulation (approximately 1%).

6. *On page 100 of the notice, the notifier states that the estimated daily intake of 2′-FL is 1.2-2.4 g/person (p)/d in OES (based on 1-2 L consumption/p/d) and 20 g/p/d in enteral feeding. Further on page 77 the notifier describes the lack of impact of the addition of 2′-FL to the osmolality of OES based on the assumption that a person can consume 1 L OES/d. Please provide:*

   - *The range of iso-osmolar concentration that is expected in the final food based on the maximum consumption of 2′-FL (2.4 g/p/d) from OES.*

   - *The range of iso-osmolar concentration that is expected in the final food based on the maximum consumption of 2′-FL (20 g/p/d) from enteral feeding.*

Osmolality is a measure of the number of particles of a substance per kilogram of solvent (mOsm/kg), whereas the osmolarity is the measure of the number of particles of a substance per liter of solution. Osmolarity is not commonly used in osmometry as it is temperature dependent: the volume of a solution can change with temperature. However, assuming that the targeted OESs and enteral feeding formulas are at room temperature, the osmolality should approximate the osmolarity of a solution. Importantly, the use of 2'-FL in OESs and enteral feeding formulas at 1.2 and 20 g/L, respectively, will result in fixed osmolarities and osmolalities of the resulting products. Therefore, the exposure to 2'-FL will depend on the amount of the OES or enteral feeding formula consumed.

As discussed in Chapter 6, Section F.3.c (pg. 77), the addition of 1.2 g 2'-FL to one liter of OES will result in an osmolarity of approximately 252.5 mOsm/L, which is comparable to the World Health Organization standard for OESs of 245 mOSm/L.

In contrast, the osmolality of enteral feeding formula typically ranges from approximately 200 to 750 mOsm/kg (Forchielli et al., 2003). Because the addition of 20 g 2'-FL/kg enteral feeding formula will add approximately 40 mOsm/kg to the final formulation and enteral feeding formula have a wide range of osmolalities, the addition of 2'-FL to the formulas will not significantly affect the overall osmolality of the final formula.

7. *The notifier provides a dietary exposure estimate to 2'-FL based on food consumption data from the 2015—2016 National Health and Nutrition Examination Survey (NHANES). We note that the notifier has provided the electrolyte solutions as separate dietary exposure estimates from the other intended uses based on the statement that the OES (such as Pedialyte) that they intend to add the 2'-FL to, will not be part of the regular diet. We note that Pedialyte can be consumed in place of other fluid replacement electrolyte solutions. Therefore, please provide a cumulative dietary exposure estimate that includes all products that could be considered under the food category of OES for this use of 2'-FL.*

As discussed in our response to Question 5, the use of 2'-FL in OESs is a stand-alone use intended to prevent dehydration caused by vomiting, diarrhea, or heat exhaustion. The intake assessment was provided separately in Chapter 3, Section 3.D.1 because of its infrequent use and the low numbers of users (i.e., 1 user) in the NHANES database (see pg. 21 of the GRAS notice). Therefore, a cumulative dietary exposure estimate that includes all products that could be considered under the food category of OES for this use of 2′-FL cannot be justified. With respect to the consideration that OES can be consumed in place of other fluid replacement electrolyte solutions, the intended use of 2'-FL in drinks intended for rehydration after exercise or travel is covered by the "sports, isotonic, and energy drinks" category in Table 7, and thus has already been included in the estimated daily intake and cumulative estimated daily intake assessments provided in Tables 8 and 9 of the Notice, respectively.

8. The vast majority of the cited studies in support of the use of 2'-FL in enteral tube feeding and OESs were conducted using long-chain nondigestible carbohydrates with a degree of polymerization much higher than 3, which is in contrast to 2'-FL (a trisaccharide). For example, PHGG has been used in many such studies; it is a galactomannan with an average MW of about 25,000-30,000 Da. This implies that the long chain nondigestible carbohydrates may be better suited in such conditions.

Please explain how the studies conducted with nondigestible carbohydrates that are chemically and structurally different from 2'-FL scientifically support the use of 2'-FL in enteral tube feeding and OESs at the proposed levels. Please also address how these studies support the general recognition of safety of the proposed use of 2'-FL.

Per GRN 000897, which received a "no questions" letter from FDA in 2020, 2'-FL is GRAS for use in enteral feeding formulas at 20 g/L, which is identical to the use level in enteral feeding formulas indicated in this GRAS Notice. Importantly, the notifier of GRN 000897 cited and relies on the same studies summarized in this GRAS Notice to support the safety and GRAS status of their 2'-FL product in enteral feeding formulas. Additionally, the studies conducted with the non-digestible carbohydrates that are chemically and structurally different from 2'-FL support the safe use of the subject of this GRAS Notice in enteral tube feeding formulas and OESs at the proposed levels because all the non-digestible carbohydrates used in the cited studies have a similar metabolic fate as 2'-FL and they were administered at levels that exceed the proposed use levels of the subject of this Notice (up to 63 g/day). Specifically, the non-digestible carbohydrates PHGG, galactomannan, and fructooligosaccharides used in the studies cited in GRN 000897 and summarized in Table 18 of this Notice are chains of carbohydrates that are resistant to the digestive enzymes in the mammalian gastrointestinal tract. As a result, they pass through the gastrointestinal tract into the large intestine relatively intact where they are fermented by anaerobic bacteria to carbon dioxide, methane, hydrogen, and short-chain fatty acids, the latter of which are readily absorbed and/or used by the colonic mucosa and other tissues for energy. The remaining, unfermented carbohydrates are then excreted in the feces. Therefore, consumers of enteral feeding formulas and OESs containing the subject of this Notice will be exposed to the same profile of metabolites as consumers of enteral feeding formulas and OES products containing other long-chain non-digestible carbohydrates or other forms of 2'-FL and Chr. Hansen has reached the same conclusion as the notifier of GRN 000897; studies conducted with long-chain nondigestible carbohydrates support the general recognition of safety of the use of 2'-FL in enteral tube feeding formulas and OESs and there is reasonable certainty that the use of 2'-FL enteral tube feeding formulas and OESs is safe.

9. Infants aged 7-12 months will be exposed to 2'-FL from a combination of infant formula and conventional foods (baby/toddler foods) increasing the dietary exposure, which is reflected in Tables 8 and 9.

Please explain how the 90th percentile dietary exposure to 2'-FL in this age group (7-12 months), which is comparable to or higher than those of some older age groups, is safe. Because the progression from 6 months of age to 7 months of age and beyond is not expected to cause an immediate remodeling of the gut architecture and gut microbiota composition, please incorporate in your discussion the perspective of gut and gut microbiota development in infants of this age group.

As discussed in Chapter 3, Section C, 2'-FL is GRAS for use in non-exempt, cow's milk-based term infant and toddler formulas; infant and toddler foods and toddler drinks, and conventional foods (GRN 000546; GRN 000571; GRN 000650; GRN 000735; GRN 000749; GRN 000852; GRN 000897; GRN 00929; GRN 000932). Chr. Hansen now intends to expand the subject of this Notice, which is already GRAS for use in infant formula and toddler formulas (GRN 000571; GRN 000929), to foods for infants and young children, meal replacements drinks for adults, non-carbonated drinks, bars, oral electrolyte solutions, and enteral tube feeding formulas. Importantly, the 2'-FL exposures in infants 0-6 and 7-12 months old that result from the intended uses and use levels of the subject of this Notice are less than those that have already been determined to be safe and GRAS for other 2'-FL products that intended for use in infant formula, toddler formulas, and conventional foods (GRN 000546; GRN 000735; GRN 000852; GRN 000932). Therefore, the safe use of the subject of this Notice for the proposed uses and use levels is similar to the safe use and use levels of other 2'-FL products that are GRAS for use in infant formula, toddler formulas, and conventional foods.

Regarding the safety of the increase 2'-FL exposure in infants aged 0 - 6 months to 7 – 12 months and older age groups, it is generally accepted that the colonization of the gastrointestinal tract with the microbiota begins at birth and expands rapidly thereafter, driven primarily by the term of pregnancy, mode of delivery, type of feeding (breast feeding vs. formula feeding), and use of antibiotics. Moreover, microbiota colonization plays an integral role in promoting the development of the gastrointestinal tract architecture and function, as well as the development and education of the host immune system, including vascular and smooth muscle development, epithelial cell development, gastrointestinal motility, intestinal barrier function, protection from enteric infections, and the gut-associated lymphoid tissue (GALT) development (reviewed in Goulet, 2015; Gensollen et al., 2016). Consistent with this, the American Academy of Pediatrics recommends "exclusive breast feeding for approximately 6 months, followed by continued breast feeding as complementary foods are introduced, with continuation of breastfeeding for 1 year or longer as mutually desired by mother and infant" (Section on Breastfeeding, American Academy of Pediatrics, 2012). Because the gastrointestinal architecture and microbiota of infants 7 – 12 months-old and older age groups are more mature than those of infants 0 - 6 months and can tolerate other foods that may contain other non-digestible carbohydrates, which have a similar metabolic fate as 2'-FL, it is reasonable to conclude that the increase in 2'-FL exposures from 0 - 6 months to 7 – 12 months and beyond is safe.

10. Both electrolytes and non-electrolytes contribute to cellular osmotic concentration and osmotic balance.

As 2'-FL is a small trisaccharide, please explain why feeding very large amounts of 2'-FL, even for limited time, will not result in osmotic imbalance and adverse effects, particularly in individuals receiving 2'-FL through OES and enteral tube feeding.

As discussed in our response to Question 6, OESs containing 2'-FL at the intended use level will have an osmolarity/osmolality that approximates the World Health Organization standard for OESs (252.5 mOsm/L vs 245 mOSm/L). Additionally, because enteral feeding formula have a wide range of osmolalities (~200 to 750 mOsm/kg) (Forchielli et al., 2003), the addition to 2'-FL to enteral feeding formulas at the intended use level will not result in enteral feeding formulas that have osmolalities outside the general range of osmolalities for enteral feeding formulas. Therefore, osmotic imbalance or adverse effects resulting from the use of 2'-FL in OESs and enteral tube feeding formulas are not expected. Importantly, enteral formulas containing 2'-FL will be used under medical supervision, with the health care provider selecting a formula with an osmolality that is appropriate for the individual's medical condition.

11. Throughout the notice, the notifier references other GRAS notices, where the subject of the notice is 2'-FL, that have been submitted to FDA and have received "no questions" letters. We evaluated GRNs 000929 and 000932 and responded in letters respectively dated, February 26, 2021, and February 18, 2021, stating that we had no questions at the time regarding the notifiers' GRAS conclusion. For the administrative record, please briefly discuss GRNs 000929 and 000932 in the context of the notifier's safety conclusion.

The 2'-FL that is the subject of GRN 000929 is manufactured using a genetically engineered strain of *Escherichia coli* and is intended for use as an ingredient in extensively hydrolyzed cow milk protein- and amino acid-based hypoallergenic exempt infant formulas for term infants and hypoallergenic toddler formulas at a level of 2 g/L of formula, as consumed. The 2'-FL that is the subject of GRN 000932 is the manufactured using a genetically engineered strain of *Corynebaterium glutamicum* and is intended to be used in milk-and soy-based, non-exempt infant formula for term infants at a maximum level of 2.4 g/L of formula as consumed, toddler formulas and variety of conventional foods intended for infants, children, and adults. Importantly, the subject of this Notice is the same as the subject of GRN 000929 and GRN 000571. In 2015, GRN 000571 established the GRAS status of the use of the subject of this Notice in non-exempt, milk-based infant formulas for term infants and toddler formulas at a maximum use level of 2 g/L of reconstituted formula. Since then, a supplement to GRN 000571 was filed to address change in the production organism and GRN 000929 was filed to expand the use of 2'-FL to extensively hydrolyzed cow milk protein- and amino acid-based hypoallergenic exempt infant formulas for term infants and hypoallergenic toddler formulas. The purpose of this Notice is to further expand the uses of the subject of GRN 000571 and GRN 000929 to conventional foods, OESs, and enteral tube feeding formulas. Importantly, the safety of the subject of GRN 000571, GRN 000929, and this Notice is supported by unpublished genotoxicology

studies, an unpublished 90-day rat oral toxicology study, and a published neonatal piglet study conducted with the subject of this Notice (Hanlon and Thorsrud, 2014), as well as published and unpublished genotoxicology, toxicology, and clinical studies conducted with other 2'-FL products. All of these studies are extensively summarized in other 2'-FL GRAS Notices, and are incorporated by reference in this GRAS notice and briefly summarized in Chapter 6. To document the GRAS status of GRN 000932, the notifier incorporated the same safety studies that were provided in this Notice, as well as a summary of the results from unpublished rats studies conducted with their own 2'-FL product. Their studies did not reveal any new adverse effects associated with the ingestion of 2'-FL. The notifiers of GRNs 000929 and 000932 also did not identify any new publicly available information that would contradict the GRAS status of the use of the subject of this Notice in conventional foods, OESs, and enteral feeding formulas. Therefore, the GRAS status of the subjects of GRN 000929 and 000932 further the support the GRAS status of the subject of this Notice.

12. *Please provide an updated literature search that discusses the safety of 2'-FL, including the date (month and year) the literature search was performed and discuss whether there are any study results that may be contradictory to a GRAS conclusion.*

As stated in "Narrative on the Conclusion of GRAS Status", Chapter 6 of the GRAS Notice (pg. 28), a literature search was conducted using Pubmed on May 10, 2021 to confirm the safe use of 2′-FL in conventional foods, OES, and enteral feeding formulas. The GRAS Determination for these uses was then conducted on May 18, 2021 and FDA was notified of the Determination on June 1, 2021. Therefore, the literature review was current at the time of the GRAS determination and FDA notification.

To further update the literature review that was conducted, an additional literature search was conducted on January 17, 2022 using Pubmed and the search terms "2-fucosyllactose AND clinical". The titles and abstracts of the retrieved studies were then reviewed to identify clinical studies that evaluated the safety of ingesting 2′-FL alone. Only one study met the inclusion criteria, Fonvig et al. (2021).

Fonvig et al. (2021) administered a daily dose of either 4.5 g of 2′-FL, 4.5 g of a mixture of 2′-FL and lacto-N-neotetraose (LNnT) (4:1), or a glucose placebo to overweight children aged 6 to 12 years for 8 weeks in a randomized, double-blinded, placebo-controlled trial. The primary endpoint was the change in fecal bifidobacteria in the intervention groups compared with placebo from baseline to the end of intervention. Secondary endpoints included the impact of HMO ingestion on gastrointestinal symptoms measured by Gastrointestinal Symptom Rating Scale (GSRS). Safety was assessed by clinical evaluation of blood chemistry and an extended panel of blood markers of inflammation (TNF alpha, IL1b, IL6, IL8, IL10, and C-reactive protein), gut barrier integrity (lipopolysaccharide-binding protein, zonulin, and haptoglobin) and metabolism (adiponectin, leptin, resistin, soluble leptin receptor, ApoA1, ApoB100, free fatty acids, and ApoB48), measurement of fecal calprotectin concentration, and collecting and monitoring adverse events (AEs) throughout the course of the trial.

Twenty-three (30.7%) of the subjects reported a total of 46 AEs between randomization and the end of intervention. Seven, 7, and 9 subjects in the placebo, 2′-FL, and 2′-FL/LNnT groups, respectively, reported at least one AE. All AEs were mild to moderate in intensity and there were no significant differences in either the intensity or incidence of AEs among the groups (13 in the placebo group, 14 the 2′-FL group, and 19 in 2′-FL/LNnT groups, respectively). GSRS scores were low at baseline for all three groups and remained low during the intervention. There were also no significant differences in the clinical chemistries, hematologies, fecal calprotectin concentrations, or markers of inflammation, gut barrier integrity, and metabolism among the groups.

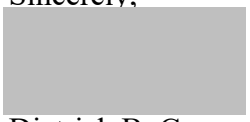
Taken together with the studies reviewed in the GRAS Notice at the time of FDA Notification, there are no results in the publicly available literature that contradict the GRAS status of the use of 2′-FL in conventional foods, OES, or enteral feeding formulas.

13. *References to "Salmonella typhimurium" on pages 35 and 36 should read Salmonella Typhimurium as serovars are not italicized. For the administrative record, please make a statement that corrects this reference.*

The references to "*Salmonella typhimurium*" on pages 35 and 36 should read "*Salmonella* Typhimurium".

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

Sincerely,



Dietrich B. Conze, Ph.D.
Managing Partner

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References

Fonvig, C.E., Amundsen, I.D., Vigsnæs, L.K., Sørensen, N., Frithioff-Bøjsøe, C., Christiansen, M., Hedley, P.L., Holm, L.A., McConnell, B., and Holm, J.C. (2021). Human Milk Oligosaccharides Modulate Fecal Microbiota and Are Safe for Use in Children With Overweight: A Randomized Controlled Trial. J Pediatr Gastroenterol Nutr 73, 408–414.

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GRN 000546 (2015). 2'-Fucosyllactose. Glycom A/S.

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GRN 000650 (2016). 2'-Fucosyllactose. Glycom A/S.

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GRN 000932 (2021). 2'-Fucosyllactose. Advanced Protein Technologies Corp.

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Section on Breastfeeding, American Academy of Pediatrics (2012). Breastfeeding and the use of human milk. Pediatrics *129*, e827–41

From: Dietrich Conze
To: Hice, Stephanie
Cc: Claire Kruger; Kathy Brailer
Subject: [EXTERNAL] Re: GRN 001014 - Chr. Hansen - 2"-FL
Date: Tuesday, April 26, 2022 3:54:32 PM

Hi Stephanie,

The notifier is in agreement to remove the use of 2'-FL in OES.

Regards.
Dietz

Dietrich Conze, PhD
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751 Rockville Pike, Unit 30-B
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> On Apr 25, 2022, at 10:39 AM, Hice, Stephanie <Stephanie.Hice@fda.hhs.gov> wrote:
>
> Dear Dr. Conze,
>
> The information provided in GRN 001014 does not support general recognition of safety for the use of 2'-fucosyllactose (2'-FL) in oral electrolyte solutions (OES) in populations with acute gastroenteritis at risk for dehydration. Further, we still have questions regarding the tolerability of this ingredient for all individuals with diarrhea/dehydration. Additionally, we evaluate ingredients intended for use in medical foods in collaboration with the Office of Nutrition and Labeling, Infant Formula and Medical Foods Staff within our Center (CFSAN/ONFL/IFMFS). Due to the issues associated with OES that can be deemed as a medical food, and the intended use of 2'-FL in this food category, we still have a few outstanding questions for the experts in ONFL/IFMFS. We cannot readily resolve these issues within the provided evaluation timeframe. To continue with the evaluation of the other intended uses included in GRN 001014, we request that the notifier remove the intended use of 2'-FL in OES. After the evaluation of GRN 001014 is complete, we suggest that the notifier request a GRAS pre-submission meeting with FDA. Such a meeting would allow the notifier to discuss the safety of the intended use of 2'-FL in OES, subsequent to our internal discussions

with ONFL/IFMFS and possible outcomes thereon.

Please contact me by close of business May 2, 2022, to let us know if the notifier is in agreement with removing the use of 2'-FL in OES. You may send this to me via email.

Sincerely,

Stiffy Hice

Stephanie (Stiffy) Hice, Ph.D. (they/them/their)
Regulatory Review Scientist & Microbiology Reviewer

Division of Food Ingredients
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
U.S. Food and Drug Administration
stephanie.hice@fda.hhs.gov

Pronouns: They-Them-Their (what is this?)

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From: Dietrich Conze
To: Downey, Jason
Cc: Claire Kruger; Kathy Brailer
Subject: [EXTERNAL] Re: Question about specifications in Chr. Hansen HMO GRNs
Date: Tuesday, May 31, 2022 2:00:04 PM

Hi Jason.

Chr. Hansen's response to your question is below.

To clarify, Chr. Hansen does indeed test for the presence of *Cronobacter* spp. using ISO 22964. So, the specifications in GRNs 1014-1017 should refer to "*Cronobacter* spp." and not "*Cronobacter sakazakii* spp." or "*Cronobacter sakazakii*". If any *Cronobacter* spp. are detected, the batch will not be released and further testing will be done to determine the exact species.

Regards.
Dietz

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> On May 26, 2022, at 12:05 PM, Downey, Jason <Jason.Downey@fda.hhs.gov> wrote:
>
> Hi Dietz,
>
> I hope you've been doing well.
>
> We have a couple of quick questions about the Cronobacter specifications in this round of Chr. Hansen HMO notices (GRNs 001014-001017). In GRN 001016, the notifier lists a specification for "Cronobacter sakazakii spp." (Table 2, page 8). The notifier states that the method used is ISO 22964. The current version of this method is ISO 22964:2017, which corresponds to "Microbiology of the Food Chain - Horizontal Method for the Detection of Cronobacter spp." For the administrative record, please clarify whether the notifier tests for the presence of Cronobacter spp. or C. sakazakii, specifically. If it is the former, please state whether presumptive positives are further analyzed to determine if the isolate is

C. sakazakii. Please clarify this for GRNs 001014-1017.

Thank you!

Jason

Jason Downey, Ph.D. (he/him/his)
Regulatory Review Scientist

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