CHR. HANSEN
improving food & health

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11/09/2021

Re: GRAS Notice for the Intended Uses of 2'-Fucosyllactose in Exempt Infant Formula for Preterm Infants Post-Discharge

Dear Dr. Carlson,

In accordance with 21 CFR Subpart E, Chr. Hansen A/S is notifying the Food and Drug Administration of their conclusion that 2'-fucosyllactose (2'-FL) produced with a genetically engineered *Escherichia coli* BL21(DE3) strain is Generally Recognized as Safe (GRAS) for its intended conditions of use in preterm post-discharge infant formula at up to 2.0 g/L, as consumed.

This 2'-FL ingredient has been previously notified as GRAS for use in other infant formula products, including non-exempt term infant formula (GRN No. 571) and exempt hypoallergenic term infant formula (GRN No. 929), at the same use level of 2.0 g/L.

Please do not hesitate to contact us should you require any clarifications regarding this GRAS notice.

Sincerely,

Manki Ho, Ph.D.
Principal Regulatory Affairs Specialist
camaho@chr-hansen.com

Cc: Katharine Urbain, Head of Regulatory Affairs – North America (uskaur@chr-hansen.com)

Generally Recognized as Safe (GRAS) Notice for the Intended Uses of 2'-Fucosyllactose in Exempt Infant Formula for Preterm Infants Post-Discharge

Chr. Hansen A/S

November 9, 2021

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Abbreviations

2'-FL	2'-Fucosyllactose
3-FL	3-Fucosyllactose
3'-SL	3'-Sialyllactose (sodium salt)
6'-SL	6'-Sialyllactose (sodium salt)
bw	Body weight
CFR	United States Code of Federal Regulations
cGMP	Current Good Manufacturing Practice
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
EDI	Estimated daily intake
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
FALCPA	Food Allergen Labeling and Consumer Protection Act of 2004
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FOS	Fructo-oligosaccharides
FSANZ	Food Standards Australia New Zealand
FSSC	Food Safety System Certification
FSIS	Food Safety and Inspection Service
GOS	Galacto-oligosaccharides
GRAS	Generally Recognized as Safe
HACCP	Hazard Analysis and Critical Control Point
HDPE	High density polyethylene
HMOs	Human milk oligosaccharides
HPAEC-PAD	High performance anion exchange chromatography coupled with pulsed amperometric detection
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kDa	kiloDalton
LNnT	Lacto-*N*-neotetraose
LNT	Lacto-*N*-tetraose
LOD	Limit of detection
LOQ	Limit of quantitation
LC-MS/MS	Liquid chromatography coupled with mass spectrometry
NOAEL	No Observed Adverse Effect Level
NMR	Nuclear magnetic resonance
pAOS	Pectin-derived acidic oligosaccharides
scFOS	Short-chain fructo-oligosaccharides
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
U.S.	United States
USDA	United States Department of Agriculture

1. Signed Statements and Certification

1.1 Statement of Intent

In accordance with the 21 CFR 170 Subpart E, Chr. Hansen A/S ("Chr. Hansen") is submitting this Generally Recognized as Safe (GRAS) Notice for their 2'-fucosyllactose (2'-FL), which is intended for use as an ingredient in exempt infant formula for preterm infants after initial hospital discharge (hereafter referred to as post-discharge).

Chr. Hansen's 2'-FL is manufactured using genetically engineered *Escherichia coli* BL21(DE3) as a processing aid[1]. The United States (U.S.) Food and Drug Administration (FDA) has previously issued a "no questions" response to the conclusion that 2'-FL obtained using this production organism is GRAS for its intended uses as an ingredient in non-exempt, milk-based term infant formula and toddler formula at a level of 2.0 g/L of formula, as consumed (GRN No. 571)[2]. A GRAS notice has also been filed for the use of this ingredient in exempt hypoallergenic infant formula for term infants and hypoallergenic formula for toddlers, which includes extensively hydrolyzed cow's milk protein- and amino acid-based formula, at a level of 2.0 g/L of formula as consumed (GRN No. 929). As indicated in their response letter to GRN No. 929, the FDA had "no questions" with regards to the conclusion of GRAS status for 2'-FL under these intended uses. Although the conditions of use for 2'-FL described within GRN No. 929 initially included exempt infant formula for preterm infants, it was agreed that a separate GRAS notice would be filed specifically for this intended use. Chr. Hansen is hereby notifying the FDA of the GRAS conclusion for the intended use of their 2'-FL in exempt infant formula for preterm infants, specifically preterm post-discharge formulas.

1.2 Name and Address of Organization

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Tel: (414) 607-5700
Fax: (414) 607-5959

1.3 Name of Notified Substance

2'-Fucosyllactose (2'-FL)

[1] This 2'-FL ingredient was initially developed by Jennewein Biotechnology GmbH, which was acquired by Chr. Hansen A/S in 2020. The legal entity (including the same company identification number), manufacturing premises, manufacturing processes, quality systems and certifications all remains the same.

[2] A GRAS notice was submitted to the FDA for the intended uses of 2'-FL produced with genetically engineered *E. coli* BL21(DE3) in cow's milk-based, non-exempt term infant formula at an increased use level of 3.64 g/L (GRN No. 924). This GRAS notice was subsequently withdrawn by the notifier.

1.4 Intended Conditions of Use

Chr. Hansen intends to use 2'-fucosyllactose (2'-FL) produced with a genetically engineered *E. coli* BL21(DE3) strain in exempt infant formula for preterm infants. At this point in time, 2'-FL is intended for use only in preterm post-discharge formula at levels up to 2.0 g/L of formula, as consumed. This use level is identical to those that have been GRAS for use in term infant formula for 2'-FL produced with the *E. coli* BL21(DE3) strain, and it is expected to yield estimated intakes of 2'-FL that are comparable to those consumed by preterm infants in the post-discharge period who are fed human breastmilk.

Breastfeeding is widely recognized as the best form of nutrition for not only term, but also preterm infants (Arslanoglu et al., 2019; Koletzko et al., 2014; Lapillonne et al., 2019). Expressed breastmilk from the mother is the first choice in preterm infant feeding, and when that is not available, donor human milk is preferred (Arslanoglu et al., 2013; Kumar et al., 2017). If mother's milk or donor human milk are not available, preterm formula should be used (Arslanoglu et al., 2013; Kumar et al., 2017). Following discharge from the hospital, infants who were born prematurely continue to be monitored to enable adequate nutritional support and ensure proper growth (Aggett et al., 2006; Lapillonne et al., 2019). Breastfeeding continues to be promoted post-discharge, though some preterm infants may require additional nutritional support, such as provision of a nutrient-enriched formula for formula-fed infants if breastfeeding is not possible (Aggett et al., 2006; Brown et al., 2020; Klein, 2002; Koletzko et al., 2014; Tudehope et al., 2013; Young et al., 2016). Post-discharge formula typically contains nutrients at levels that are intermediary between preterm formula and standard term formula (Klein, 2002; Tudehope et al., 2013; Young et al., 2016).

There are ongoing efforts to develop formula products that are matched closely to that of human breastmilk. One key compositional difference between commercialized infant formula and human breastmilk is that the latter contains a highly abundant and unique fraction of structurally diverse glycans known as human milk oligosaccharides (HMOs) (Bode, 2012). HMOs represent the third largest component of breastmilk solid matter after lactose and lipids, with 2'-FL being one of the most abundant glycans present (Castanys-Muñoz et al., 2013; Coppa et al., 2004; Soyyılmaz et al., 2021). While HMOs represent a large component of human breastmilk, they occur only at very low concentrations in cow's milk, which is commonly used to formulate infant formula (Albrecht et al., 2014). Accordingly, manufactured versions of purified HMOs have been widely commercialized as ingredients in infant formula. A number of GRAS notices have been filed for this intended use of 2'-FL (GRN Nos. 546, 571, 650, 735, 749, 852, 859, 897, 924, 929, 932, 987) and other HMO ingredients, including a mixture of 2'-fucosyllactose and difucosyllactose (GRN No. 815), 3-fucosyllactose (GRN Nos. 925, 951), lacto-*N*-tetraose (GRN Nos. 833, 923), 3'-sialyllactose sodium salt (GRN Nos. 766, 880, 921), 6'-sialyllactose sodium salt (GRN Nos. 881, 922), and lacto-*N*-neotetraose (GRN Nos. 547, 659, 895, 919).

As with most term infant formula, preterm post-discharge formulas are typically formulated using cow's milk, and therefore do not contain HMOs. Accordingly, the addition of 2'-FL to preterm post-discharge formula would help bring the compositional profile closer to that of human breastmilk, similar to the intended uses of 2'-FL in term infant formula.

1.5 Statutory Basis for GRAS Conclusion

Pursuant to the GRAS rule [81 Fed. Reg. 159 (17 August 2016)], Chr. Hansen has concluded that the intended use of 2'-FL in exempt preterm post-discharge infant formula, as described herein, is GRAS through scientific procedures, in accordance with 21 CFR §170.30 (a) and (b).

1.6 Premarket Approval Status

It is the view of Chr. Hansen that 2'-FL is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, based on our conclusion that the notified substance is GRAS under the conditions of its intended use.

1.7 Availability of Information

The data and information that serve as the basis for the conclusion that the intended use of Chr. Hansen's 2'-FL is GRAS will be made available to the FDA upon request. Chr. Hansen will allow the FDA to review and copy the data and information at the below address during customary business hours. Alternatively, Chr. Hansen will provide the FDA with a complete copy of the data and information that are the basis for the conclusion of the GRAS status, either in an electronic format that is accessible for the FDA's evaluation, or on paper.

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1.8 Freedom of Information Act

None of the data and information contained in this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

1.9 Certification

To the best of our knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the intended use of 2'-FL.

1.10 FSIS Statement

Not applicable. 2'-FL is not intended for use in products subject to regulation by Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture (USDA).

1.11 Name, Position and Signature of Responsible Person

November 9, 2021

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November 9, 2021

Katharine Urbain
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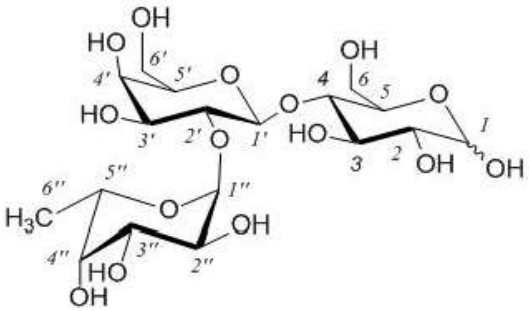
Date

2. Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

Common or Usual Name:	2'-Fucosyllactose (2'-FL)
Chemical Name:	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
CAS Number:	41263-94-9
Molecular Weight:	488.439 g/mol
Molecular Formula:	$C_{18}H_{32}O_{15}$
Structural Formula:	

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 (Soyyılmaz et al., 2021; Urashima et al., 2012). Chr. Hansen manufactures 2'-FL through fermentation using a genetically engineered *E. c*oli BL21(DE3) strain as a processing aid. The resulting ingredient is identical to the material that has been described in GRN No. 571 and GRN No. 929. The identity and purity of 2'-FL produced by fermentation has been confirmed by proton and carbon nuclear magnetic resonance spectroscopy (^{1}H and ^{13}C NMR), high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD), liquid chromatography-tandem mass spectrometry (LC-MS/MS), and optical rotation analysis. Details of these analyses are available in GRN No. 571 and the Supplement to GRN No. 571.

In brief, the structure of 2'-FL obtained by fermentation is confirmed to be chemically and structurally equivalent to the 2'-FL naturally present in human breastmilk. The purified spray dried powder consists of a minimum of 90% 2'-FL on a dry weight basis. The 2'-FL powder also contains small amounts of other residual carbohydrates that occur naturally in human milk (lactose, difucosyllactose, 3-fucosyllactose, fucose, glucose and galactose) (Asakuma et al., 2008; Thurl et al., 1996), as well as fucosylgalactose, an oligosaccharide breakdown product that occurs naturally in the human body (Chester et al., 1979). These carbohydrates are present at concentrations of ≤5% each.

2.2 Method of Manufacture

The production process for Chr. Hansen's 2'-FL has been described in detail in GRN No. 571, with minor modifications introduced in a Supplement to GRN No. 571 and in GRN No. 929. Information relating to the production process for 2'-FL that were presented in those GRAS notices is incorporated by reference herein.

2.2.1 Production Strain

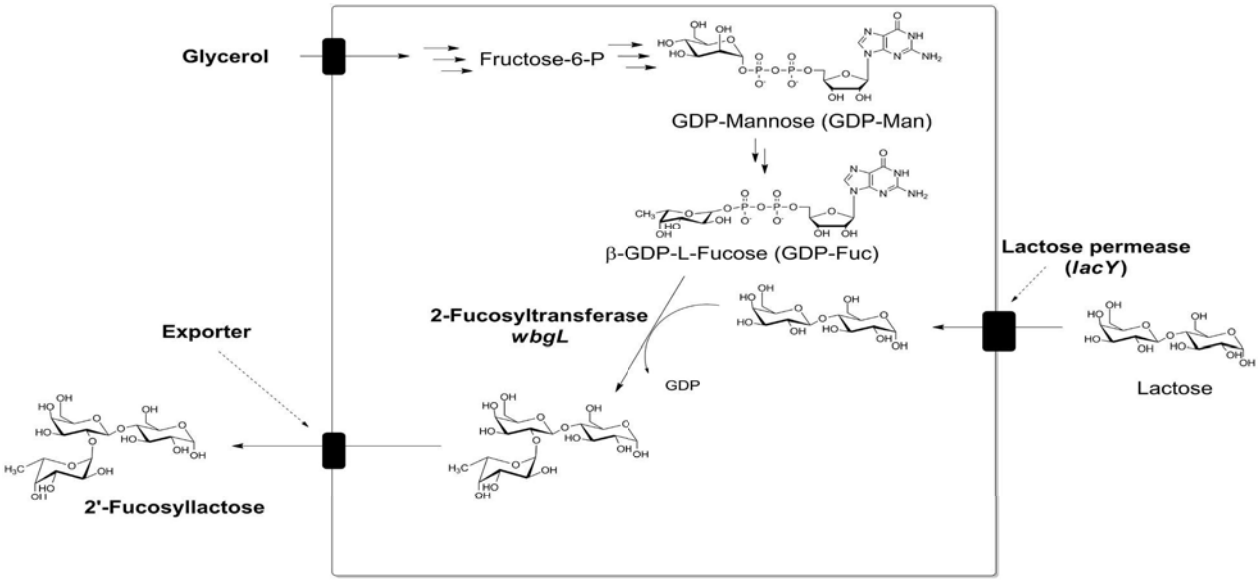
2'-FL is manufactured by fermentation using a genetically engineered strain of *E. coli* BL21(DE3) as a processing aid. The parental organism, *E. coli* BL21(DE3), is a safe, non-pathogenic commensal bacterium that is often used for the production of various industrial, pharmaceutical, and food biotechnology preparations (see Section 6.2). The taxonomic classification of *E. coli* BL21(DE3) is presented in Table 2.2.1-1.

Table 2.2.1-1 Taxonomic Classification of the Parental Organism

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

Details of the modifications introduced into *E. coli* BL21(DE3) to allow for the production of 2'-FL are available in GRN No. 571 and its accompanying Supplement. The production strain is modified to increase the import of lactose, and to enhance the *de novo* biosynthesis of GDP-L-fucose. The GDP-L-fucose is used as a substrate, along with lactose, to produce 2'-FL by a heterologous 2-fucosyltransferase. The 2'-FL is then exported from the cell by the overexpression of a sugar efflux exporter, allowing 2'-FL to be obtained from the culture broth. A schematic overview of the biosynthetic pathway for 2'-FL in the genetically engineered strain of *E. coli* BL21(DE3) is presented in Figure 2.2.1-1.

Figure 2.2.1-1 Outline of the Metabolic Pathway for 2'-Fucosyllactose Synthesis in Genetically Engineered *E. coli* BL21(DE3)



A Supplement to GRN No. 571 was submitted to the FDA in 2019, to which the FDA has issued a "no questions" letter (see Appendix A). This Supplement informed the FDA of a minor change in the manufacturing process, specifically with respect to the production strain employed. Instead of the *E. coli* BL21(DE3) #1540 strain that had been described in GRN No. 571, 2'-FL may alternatively be produced using its parental strain, which is denoted *E. coli* BL21(DE3) #1242 or *JBT-2FL⊿lacZ*. Strain #1242 contains the same genetic components as strain #1540, but it lacks the ability to degrade lactose. The production strain does not contain plasmids or other episomal vectors and is not capable of DNA transfer to other organisms. The *E. coli* BL21(DE3) #1242 production stain has been deposited at DSMZ - German Collection of Microorganisms and Cell Cultures GmbH with the deposition number DSM 33609.

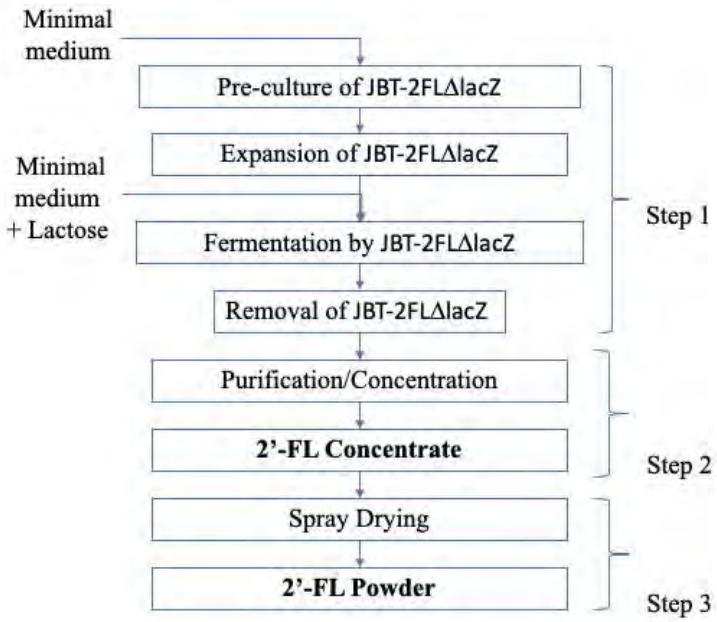
2.2.2 Manufacturing Process

A flowchart of the manufacturing process is presented in Figure 2.2.2-1. Batch fermentation is performed in a minimal medium containing a simple, pure carbon source (e.g., glucose or glycerol) and the lactose substrate. Additionally, the major constituents of the fermentation medium include ammonium dihydrogen phosphate ($NH_4H_2PO_4$), dipotassium phosphate (K_2HPO_4), citric acid, potassium hydroxide (KOH), and magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$). No antibiotics or inhibitors are used during the fermentation process.

During fermentation, 2'-FL is produced and secreted into the culture medium. The fermentation process continues until a certain level of 2'-FL is obtained. Since the production strain does not have the ability to degrade excess lactose, a food-grade commercial lactase may be added if excess lactose is present in the media. The culture supernatant containing 2'-FL is isolated from the medium and the microbial biomass is removed *via* 10 kDa cross flow filtration. The filtrate is subjected to a series of cationic and

anionic ion exchange resins to remove impurities (*e.g.*, proteins, DNA, organic acids, and inorganic salts). The eluent containing 2'-FL is then concentrated by evaporation and subjected to multiple purification steps to decolorize and further remove impurities, including treatment with activated carbon, electrodialysis, ion exchange chromatography, and ultrafiltration. Lastly, the resulting 2'-FL concentrate is spray dried to generate powdered 2'-FL.

Figure 2.2.2-1 Production Process for 2'-Fucosyllactose



2.2.3 Processing Aids and Food Contact Substances

All raw materials, processing aids, and food contact substances used to produce 2'-FL are the same as those used to produce the 2'-FL that is the subject of GRN No. 571, except that cobalt chloride is no longer used in the culture medium. All materials employed are food-grade and suitable for their use, as described in GRN No. 571 and incorporated by reference herein.

2.2.4 Quality Program

All manufacturing is done in accordance with current good manufacturing practices (cGMP) consistent with 21 CFR Parts 110 and 117. All Chr. Hansen plants have fully implemented HACCP plans, standard operating procedures and quality control programs to ensure quality of the product being produced. Each plant complies with a set of basic GMP rules, also called Pre-Requisite Program (PRP) according to Chr. Hansen's Quality, GMPs and Food Safety Principles, which are publicly available from our website www.chr-hansen.com. As part of the HACCP plan, each manufacturing process has appointed an OPRP (Operational Pre-Requisite Program) and CCPs (Critical Control Points). The OPRP and CCP's are documented and classified as specifically critical for the safety of food ingredients produced in the plant. All Chr. Hansen facilities manufacturing final products maintain FSSC 22000 certification.

2.2.5 Allergen Control

Chr. Hansen controls for all allergens listed in Regulation (EU) No 1169/2011 and the U.S. Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA). No allergenic materials as listed in Regulation (EU) No 1169/2011 and FALCPA are employed in the production of Chr. Hansen's 2'-FL, other than lactose from cow's milk. Chr. Hansen communicates the allergen status of our products in accordance with these two regulations. Allergen control is managed *via* our GMP and HACCP programs that are FSSC 22000 certified at all of our production sites. Allergen communication is managed via our Quality Management and HACCP programs that are ISO 22000 certified.

2.3 Specifications and Analytical Data

To ensure that a consistent food-grade material is produced, Chr. Hansen has established specifications for their 2'-FL ingredient. The physical, chemical, and microbiological specifications for 2'-FL are presented in Table 2.3-1. The ingredient is specified to contain ≥90% of 2'-FL on a dry weight basis, with small amounts of residual carbohydrate by-products, including lactose (≤5%), 3-fucosyllactose (≤5%), difucosyllactose (≤5%), fucosylgalactose (≤3%), glucose (≤3%), galactose (≤3%), and fucose (≤3%). Limits are also included to ensure the absence of endotoxins, aflatoxin M1, recombinant DNA from the production strain, heavy metals, and microbiological contaminants. For the purposes of batch testing, the Bradford method is used to analyze for the presence of proteins, which has a limit of quantification of ≤10 µg/g. As described in GRN No. 929, the absence of protein in the 2'-FL ingredient has been demonstrated with more sensitive analytical techniques (*i.e.*, SDS-PAGE with a limit of detection of 10 µg/kg).

Each specification parameter is measured using the same compendial and/or internally validated, fit-for-purpose methods that were provided in GRN No. 571. Importantly, since the filing of GRN No. 571 and the GRN No. 571 supplement, 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. These limits are considered sufficient to produce safe food ingredients. Aside from these changes, all other specification parameters and acceptable limits remain the same. These specifications listed in Table 2.3-1 are identical to those presented in GRN No. 929. Data from five batches of powdered 2'-FL show that the manufacturing process continues to reproducibly produce a product that meets the established specifications (see Table 2.3-1).

Table 2.3-1 Specifications and Batch Analysis Data for 2'-FL Powder

Parameter	Analytical method	Specification	Batch number				
			16130039	16116049	16151039	26108010	26120020
Physical Parameters							
Appearance (Color)[1]	Visual	White to ivory-colored	Complies	Complies	Complies	Complies	Complies
Appearance (Form)[1]		Spray-dried powder	Complies	Complies	Complies	Complies	Complies
Chemical Parameters							
2'-Fucosyllactose	HPAEC-PAD[2]	≥ 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

Parameter	Analytical method	Specification	Batch number				
			16130039	16116049	16151039	26108010	26120020
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[1]	Nanoquant (modified Bradford)	≤ 100 µg/g	< 10	< 10	< 10	< 10	< 10
Ash[3]	ASU L 06.00-4	≤ 0.5 %	< 0.01	0.03	0.08	< 0.01	0.08
Moisture[1]	KF titration	≤ 9.0 %	5.8	5.8	6.3	6.6	5.2
Endotoxins[4]	Ph. Eur. 2.6.14	≤ 300 EU/g	14	< 5	< 5	< 5	< 5
Aflatoxin M1[3]	DIN EN ISO 14501	≤ 0.025 µg/kg	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
GMO residues[5]	qPCR	Negative	Negative	Negative	Negative	Negative	Negative
Heavy Metals							
Arsenic[3]	ASU L 00.00-135 – ICP-MS	≤ 0.2 mg/kg	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Cadmium[3]		≤ 0.1 mg/kg	< 0.010	< 0.010	< 0.010	< 0.010	< 0.010
Lead[3]		≤ 0.02 mg/kg	< 0.010	< 0.010	0.020	< 0.010	< 0.010
Mercury[3]		≤ 0.5 mg/kg	< 0.005	0.007	< 0.005	< 0.005	< 0.005
Microbiological Criteria							
Standard Plate Count[3]	ISO 4833-2	≤ 10000 cfu/g	< 10	< 10	30	20	< 10
Yeast and Mold[3]	ISO 21527-2	≤ 100 cfu/g	< 20	< 20	< 20	< 20	< 20
Coliform	ISO 4832	Absent/11 g	Absent	Absent	Absent	Absent	Absent
Enterobacteriaceae[3]	ISO 21528-1	Absent/11 g	Absent	Absent	Absent	Absent	Absent
Salmonella[3]	ISO 6579	Absent/25 g	Absent	Absent	Absent	Absent	Absent
Cronobacter sakazakii[3]	ISO/TS 22964	Absent/10g	Absent	Absent	Absent	Absent	Absent

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

[1] Determined by Chr. Hansen A/S using internally validated methods. Protein LOQ = 10 µg/g.

[2] Carbohydrate by-products with a percent area greater than 0.5% (limit of quantitation) are considered.

[3] 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 mg/kg; aflatoxin M1 LOQ = 0.025 µg/kg.

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

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

2.4 Stability

2.4.1 Genetic Stability of the Production Strain

As detailed in GRN No. 571, to ensure genomic stability and finished product batch-to-batch consistency, all modifications that were introduced into the genetically engineered *E. coli* BL21(DE3) 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. Moreover, the production strain is stored as glycerol stocks in a master cell bank at -80°C, which are used to produce the working cell banks employed for the manufacture of 2'-FL.

2.4.2 Stability of 2'-FL Powder

As described in GRN No. 571, Chr. Hansen's spray dried 2'-FL powder 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.

3. Dietary Exposure

3.1 Overview

Chr. Hansen intends to use their 2'-FL in preterm post-discharge formulas at levels up to 2.0 g/L of formula, as consumed. This use level is identical to those that have been GRAS for use in term infant formula for 2'-FL produced with a genetically engineered *E. coli* BL21(DE3) strain, and it is well within the concentrations of 2'-FL that have been reported in human breastmilk, following either term or preterm births, as explained in Section 3.2 below. An estimation of the intake of 2'-FL occurring in preterm infants post-discharge who are fed human milk is also presented in Section 3.2. The estimated daily intake of 2'-FL from its intended uses in preterm post-discharge formulas are then presented in Section 3.3. Overall, the estimated intakes of 2'-FL from its intended uses in preterm post-discharge formulas are not expected to exceed those consumed by preterm infants who are fed human milk, which help to support the safety of its use.

3.2 History of Safe Consumption by Preterm Infants Fed Human Milk

3.2.1 Concentrations of 2'-FL in Human Milk

Total concentrations of HMOs are reported in the ranges of 20 to 25 g/L in colostrum, and up to 20 g/L in mature human milk (Bode, 2012). Maternal genetic factors (*i.e.*, allelic variations in the Secretor and Lewis genes) is a key determinant of the HMO composition of human milk, though other factors (such as lactation stage) may also play a role (Han et al., 2021; Walsh et al., 2020). 2'-FL is one of the most abundant oligosaccharides in human milk (Castanys-Muñoz et al., 2013; Coppa et al., 2004; Soyyılmaz et al., 2021). It belongs to the group of fucosylated HMOs, which constitute between 50 to 80% of the total HMO fraction in human milk from the majority of lactating women (Bode, 2012). Approximately 80% of women express the α-1,2-fucosyltransferase enzyme responsible for fucosylating lactose at the 2'-O-position in the mammary gland (*i.e.*, "secretors"), and therefore produce breastmilk containing 2'-FL

(Castanys-Muñoz et al., 2013; EFSA NDA Panel, 2015; R. M. Erney et al., 2000; FSANZ, 2019; McGuire et al., 2017).

In a systematic review conducted by Thurl et al. (2017), the mean concentration of 2'-FL in the breastmilk of secretor mothers who delivered preterm was reported at **2.77 g/L** (95% confidence limit: 0.76 to 4.78 g/L), which is similar to the mean concentration of **2.74 g/L** (95% confidence limit: 2.43 to 3.04 g/L) reported for the breastmilk of secretor mothers who delivered at term (see Table 3.2.1-1). The authors noted that: "*Although the data analyses with term and preterm milks were conducted separately in this review, no clear effects of gestational age on HMOS concentrations were found*" (Thurl et al., 2017).

One recent longitudinal study compared the HMO composition of human milk at equivalent lactation stages and postmenstrual age (Austin et al., 2019). This study involved 500 samples of milk from 28 mothers of term infants born at 37 0/7 weeks to 41 6/7 weeks gestation, and 25 mothers of preterm infants born at 28 0/7 weeks to 32 6/7 weeks gestation. Samples were collected once weekly at intervals of 7±1 days during the first 8 weeks after both preterm and term deliveries, with additional samples collected from mothers with preterm births at intervals of 14±1 days until 16 weeks after delivery. Similar to previous studies (Kunz et al., 2017; Thurl et al., 2017), the concentrations of HMOs in human milk was generally comparable between term and preterm groups at equivalent lactation stages (*i.e.*, at equivalent postpartum age). However, since HMO concentrations tend to decline over the course of lactation, at equivalent developmental ages (*i.e.*, postmenstrual age), the concentrations of 2'-FL in preterm milks was reported to be significantly lower than term milks at postmenstrual age of weeks 39 to 43, and at week 45, amongst mothers of Milk Group 1 (*i.e.*, mothers with active FUT2 and FUT3 enzymes). Nonetheless, the concentrations of 2'-FL in preterm milks at these postmenstrual ages, which are presented below in Table 3.2.1-1, continue to be within the intended use level of 2 g/L for 2'-FL in preterm post-discharge formula. For instance, the maximum values reported for 2'-FL in preterm milks were as high as **3.6 g/L** between weeks 37 to 48 postmenstrual age.

Table 3.2.1-1 Concentration of 2'-FL in Human Milk of Secretor Mothers Following Preterm and Term Births (Adapted from Austin et al., 2019)

Gestation	Postmenstrual Age	n	2'-FL Concentrations (g/L)[1]					
			Mean	SD	Median	Q1	Q3	Max
Preterm	30 weeks	7	2.9	1.3	3.4	2.9	3.5	4.0
	31 weeks	9	2.1	1.0	2.3	2.0	2.4	3.8
	32 weeks	13	2.3	1.2	2.1	1.7	3.1	4.2
	33 weeks	18	2.4	1.2	2.4	1.6	2.7	5.5
	34 weeks	19	2.0	0.9	1.8	1.5	2.8	3.6
	35 weeks	18	1.8	0.7	1.8	1.6	2.3	3.0
	36 weeks	19	1.9	0.8	1.8	1.7	2.4	3.1
	37 weeks	18	1.9	0.8	1.8	1.5	2.3	3.3
	38 weeks	11	2.1	0.8	2.2	1.8	2.7	3.0
	39 weeks	16	1.8	0.8	1.7	1.3	2.5	3.2
	40 weeks	7	2.1	0.8	1.7	1.6	2.7	3.4

	Postmenstrual Age	n	2'-FL Concentrations (g/L)[1]					
			Mean	SD	Median	Q1	Q3	Max
	41 weeks	12	2.0	1.0	1.9	1.4	2.5	3.6
	42 weeks	6	1.8	0.8	1.5	1.4	2.5	2.9
	43 weeks	10	1.6	0.8	1.7	1.2	2.1	2.8
	44 weeks	5	1.7	0.8	1.5	1.3	2.1	2.7
	45 weeks	9	1.4	0.7	1.4	0.9	2.1	2.2
	46 weeks	6	1.8	0.9	1.7	1.3	2.5	2.9
	47 weeks	3	1.8	0.7	2.1	1.5	2.2	2.2
	48 weeks	4	1.8	1.2	1.5	1.3	2.0	3.5
Term	38 weeks	2	3.7	0.7	3.7	3.4	4.0	4.2
	39 weeks	9	3.8	1.4	3.5	2.6	5.0	5.6
	40 weeks	13	3.0	0.7	3.1	2.3	3.4	4.3
	41 weeks	21	2.9	1.0	2.8	2.1	3.2	5.0
	42 weeks	21	2.6	0.7	2.5	2.0	3.1	3.9
	43 weeks	21	2.5	0.8	2.5	1.7	2.9	4.2
	44 weeks	20	2.3	0.7	2.2	1.6	3.0	3.4
	45 weeks	21	2.2	0.9	2.2	1.7	3.0	4.0
	46 weeks	17	2.1	0.8	2.2	1.6	2.7	3.1
	47 weeks	12	2.2	0.8	2.3	1.5	2.9	3.1
	48 weeks	6	2.6	0.7	2.8	2.3	3.0	3.2

Abbreviations: SD, standard deviation; Q1, quartile 1 (25th percentile); Q3, quartile 3 (75th percentile).
[1] Adapted from Supplementary Table 4 of Austin et al., (2019).

3.2.2 Estimated Daily Intake of 2'-FL in Preterm Infants Receiving Human Milk

A number of authoritative guidelines have been published on the nutrient requirements of preterm infants, which include estimations of the fluid volumes consumed on a daily basis. For instance, the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) considers **200 mL/kg body weight (bw)/day** to be a "reasonable upper limit" of fluid intake, and 135 ml/kg body weight/day to be the minimum volume, for the enteral intake of stable-growing preterm infants up to a weight of 1,800 g (Agostoni et al., 2010). ESPGHAN further noted that: "*For routine feeding, rates of **150 to 180 mL·kg^{-1}·day^{-1}** nutrient intake when standard formula or fortified breast milk is used are likely to achieve meeting nutrient requirements*" (Agostoni et al., 2010). Likewise, an *ad hoc* Expert Panel convened by the Life Sciences Research Office of the American Society for Nutritional Sciences under contract with the U.S. FDA based their recommendations for the nutrient content of preterm infant formula assuming fluid intakes of **150 mL/kg bw/day** (Klein, 2002).

The recommendations in these guidelines are consistent with the levels of human milk intakes that have been reported for preterm and term infants in the literature. A recent comprehensive review evaluated publications measuring human milk intake in term infants (28 studies) and preterm infants (7 studies)

(Yeung et al., 2020). Mean daily weight-normalized human milk intake was reported to increase starting from birth, reaching a maximum of 152.6 mL/kg bw/day at 19.7 days of postnatal age and then declining thereafter. On a body weight-normalized basis, the study authors noted that preterm infants do not present a substantial difference in feeding volume trajectories across ages when compared with term infants. Similarly, in a study of preterm infants in the post-discharge period, the mean volume of intake was reported at 190 mL/kg bw/day at an age equivalent to term birth (*i.e.*, 40 weeks after the last menstrual period), and gradually declining to 103 mL/kg bw/day by 9 months corrected age (Carver et al., 2001).

Based on these estimates of milk consumption volumes, and the concentrations of 2'-FL that have been reported in human milk (see Section 3.2.1 above), an estimation of the intakes to 2'-FL can be derived for preterm infants, as summarized in Table 3.2.2-1. For comparison, the estimated intake of 2'-FL from human milk for term infants that had been derived by the EFSA NDA Panel (EFSA NDA Panel, 2019), which were calculated using higher concentrations of 2'-FL, is summarized in Table 3.2.2-2.

Table 3.2.2-1 Estimated Daily Intake of 2'-FL from Human Milk by Preterm Infants

Volume of Milk Intake	Concentration of 2'-FL in Human Milk[1]		Estimated Daily Intake of 2'-FL from Human Milk
135 mL/kg bw/day	Mean:	2.77 g/L	374 mg/kg bw/day
	High:	3.6 g/L	486 mg/kg bw/day
150 mL/kg bw/day	Mean:	2.77 g/L	416 mg/kg bw/day
	High:	3.6 g/L	540 mg/kg bw/day
200 mL/kg bw/day	Mean:	2.77 g/L	554 mg/kg bw/day
	High:	3.6 g/L	720 mg/kg bw/day

Abbreviation(s): bw, body weight.
[1] The mean level (2.77 g/L) is the value derived by Thurl et al. (2017) for preterm milk. The high level (3.6 g/L) represents the maximum concentration of 2'-FL detected in preterm milk at postmenstrual age of 41 weeks, as reported by Austin et al. (2019).

Table 3.2.2-2 Estimated Daily Intake of 2'-FL from Human Milk by Term Infants, as Derived by the EFSA NDA Panel

Volume of Fluid Intake[1]	Concentration of 2'-FL in Human Milk[2]		Estimated Daily Intakes to 2'-FL from Human Milk[3]
800 mL/day	Mean:	2.38 g/L	284 mg/kg bw/day
	High:	4.78 g/L	571 mg/kg bw/day
1,200 mL/day	Mean:	2.38 g/L	426 mg/kg bw/day
	High:	4.78 g/L	856 mg/kg bw/day

Abbreviation(s): bw, body weight.
[1] In their Scientific Opinion on the nutrient requirements for infants and young children, the EFSA NDA Panel considers the average volume of breastmilk consumed to be 800 mL/day with an upper bound of 1,200 mL/day (EFSA NDA Panel, 2013).
[2] The EFSA NDA Panel used the 2'-FL concentrations in human milk that had been reported in Erney et al. (2001).
[3] Derived by the EFSA NDA Panel based on the assumption of 6.7 kg bw.

3.3 Estimated Daily Intakes of 2'-FL from its Intended Uses

3.3.1 Exclusively Formula-Fed Infants

Similar to formula for term infants, preterm post-discharge formula provides the sole source of nutrition for exclusively formula-fed infants, and therefore will provide the only source of supplemental 2'-FL in the diet until complementary foods are introduced. In infants receiving a combination of human milk and formula, the overall intake to 2'-FL is expected to remain comparable to those receiving formula only (or alternatively, human milk only), given that the intended use level of 2.0 g/L reflects the range of 2'-FL concentrations normally found in breast milk (see Section 3.2).

A conservative estimate of the mean and high (95th percentile) consumption levels of infant formula has been derived as **200 mL/kg bw/day** and **260 mL/kg bw/day**, respectively, by the European Food Safety Authority (EFSA) Scientific Committee (EFSA Scientific Committee et al., 2017). The high consumption value of 260 mL/kg bw/day is considered appropriate for use in the risk assessment of substances which do not accumulate in the body that are present in foods intended for infants below 16 weeks of age, including preterm infants on enteral (formula) feeding (EFSA Scientific Committee et al., 2017). Similar estimations on the volume of formula consumed daily on a body weight basis have also been employed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for the risk assessment of substances for use in infant formulas (including formulas for special medical purposes intended for infants), such as pectin and octenyl succinic acid (OSA)-modified starch, as examples (Constable et al., 2017; JECFA, 2015, 2017).

The estimated daily intake for 2'-FL from its intended use in preterm post-discharge formula, based on accepted estimations of formula intakes, is presented in Table 3.3.1-1. It is notable that the mean and high levels of formula intakes derived by EFSA are considered to be conservative estimates. In practice, a more realistic intake volume for preterm post-discharge formula is expected to be **150 mL/kg bw/day**. Across all exposure scenarios, the estimated daily intake of 2'-FL from its intended uses in post-discharge formula (*i.e.*, up to **520 mg/kg bw/day**) remain within those of infants (both preterm and term) fed human milk, as described above in Section 3.2.2.

Table 3.3.1-1 Estimated Daily Intake to 2'-FL in Formula-Fed Preterm Infants Post-Discharge

Maximum Use Level for 2'-FL	Estimated Consumption Volumes of Preterm Post-Discharge Formula	Estimated Daily Intake to 2'-FL from its Intended Uses in Preterm Post-Discharge Formula
2.0 g/L	150 mL/kg bw/day[1]	300 mg/kg bw/day
	200 mL/kg bw/day[2]	400 mg/kg bw/day
	260 mL/kg bw/day[2]	520 mg/kg bw/day

Abbreviation(s): bw, body weight.

[1] Represents the typical level of formula intake that have been reported (WHO, 2009).

[2] Represents the mean (200 mL/kg bw/day) and high (260 mL/kg bw/day) consumption levels of infant formula considered appropriate for use in the risk assessment of substances present in foods intended for infants below 16 weeks of age, including preterm infants on enteral (formula) feeding (EFSA Scientific Committee et al., 2017).

3.3.2 Combined Intakes from GRAS Uses of 2'-FL in Other Food Products

Many authoritative guidelines recommend exclusive breastfeeding for the first six months of age and continued breastfeeding with complementary foods up to two years of age (Eidelman & Schanler, 2012; Kramer & Kakuma, 2012; Pound et al., 2012; WHO, 2021). In cases where breastfeeding is not possible, infant formula is considered a suitable alternative. For formula-fed preterm infants, switching to a standard term formula is typically recommended once they have reached their birth centile (*i.e.*, after catch-up has been attained) (Kumar et al., 2017). It is possible that infants may continue to consume the nutrient-enriched preterm post-discharge formula even upon the introduction of complementary foods. In such case, the intended uses of Chr. Hansen's 2'-FL in preterm post-discharge formula is considered substitutional to the existing uses of 2'-FL in term infant formula, which is GRAS at up to 2.4 g/L in the U.S. Thus, no material increase in dietary exposure is expected, and the estimated daily intake of 2'-FL from its intended uses in preterm post-discharge formula, amongst infants who may consume 2'-FL from other current food uses in the U.S., is expected to be within those estimated previously for term infants age up to 12 months of age.

Table 3.3.2-1 Estimated Daily Intake of 2'-FL in Infants from its Intended Uses Described in GRAS Notices Issued "No Questions" Responses by the U.S. FDA

GRN No.	Intended Uses of 2'-FL[1,2]		Method of Exposure Assessment	90th Percentile EDI for Infants (12 Months of Age) and Toddlers	
	Infant Formula	Other Foods		g/day	mg/kg bw/day
929	Exempt hypoallergenic IF for term infants at 2.0 g/L.	Hypoallergenic toddler formula at 2.0 g/L.	NHANES 2015-2016[3]	0 to 5 mo: 2.6	0 to 5 mo: 403
				6 to 11 mo: 2.9	6 to 11 mo: 320
				12 to 35 mo: 1.4	12 to 35 mo: 130
897	Non-exempt IF for term infants at 2.4 g/L.	Toddler formulas at 2.4 g/L; infant and toddler foods at 12 g/kg; and toddler drinks at 1.2 g/L. Also includes other conventional foods and enteral formulas (≥11 years old) at 1.2 to 40 g/kg.	Exposure assessment for infants and toddlers was IbR from GRN No. 749	IbR from GRN No. 749	IbR from GRN No. 749
852	Non-exempt IF for term infants at 2.4 g/L.	Toddler formulas at 2.4 g/L; infant and toddler foods at levels ranging from 0.24 to 1.2 g/serving. Also includes other conventional foods at levels ranging from 0.28 to 1.2 g/serving.	IbR from GRN No. 735	IbR from GRN No. 735	IbR from GRN No. 735

GRN No.	Intended Uses of 2'-FL[1,2]		Method of Exposure Assessment	90th Percentile EDI for Infants (≤12 Months of Age) and Toddlers	
				g/day	mg/kg bw/day
749	Non-exempt IF for term infants at 2.4 g/L.	Toddler formulas at 2.4 g/L; infant and toddler foods at 12 g/kg; and toddler drinks at 1.2 g/L.	NHANES 2009-2010, 2011-2012	*EDI from all intended uses (IF, infant and toddler foods, toddler drinks)*	
				0 to 6 mo: 5.29	0 to 6 mo: 712
				7 to 12 mo: 8.36	7 to 12 mo: 987
				13 to 36 mo: 1.97	13 to 36 mo: 146
				EDI from IF only	
				0 to 6 mo: 2.91	0 to 6 mo: 536
				7 to 12 mo: 2.63	7 to 12 mo: 296
				1 to 3 years: 1.41[4]	1 to 3 years: 117[4]
735	Non-exempt IF for term infants at 2.4 g/L.	Toddler formulas at 2.4 g/L; infant and toddler foods at levels ranging from 0.24 to 1.2 g/serving. Also includes other conventional foods at levels ranging from 0.28 to 1.2 g/serving.	NHANES 2013-2014	*EDI from all intended uses (including foods for the general population)*	
				0 to 5 mo: 3.00	0 to 5 mo: 532
				6 to 11 mo: 3.86	6 to 11 mo: 447
				12 to 35 mo: 2.97	12 to 35 mo: 243
				EDI from IF only	
				0 to 5 mo: 2.88[4]	0 to 5 mo: 498[4]
				6 to 11 mo: 2.56	6 to 11 mo: 311
				12 to 35 mo: 1.14[4]	12 to 35 mo: 101[4]
650	Non-exempt IF for term infants at 2.4 g/L.	Toddler formulas at 2.4 g/L; infant and toddler foods, and other conventional foods at levels ranging from 0.084 to 2.04 g/serving.	Exposure assessment for infants and toddlers was IbR from GRN No. 546	IbR from GRN No. 546	IbR from GRN No. 546
571	Non-exempt IF for term infants at 2.0 g/L.	Toddler formulas at 2.0 g/L.	NHANES 2009-2010	0 to 5 mo: 2.5	NR
				6 to 11 mo: 2.2	NR
				12 to 35 mo: 2.0	NR
546	Non-exempt IF for term infants at 2.4 g/L.	Toddler formulas at 2.4 g/L; infant and toddler foods, and other conventional foods at levels ranging from 0.084 to 2.04 g/serving.	NHANES 2009-2010	*EDI from IF and intended food uses*	
				0 to 6 mo: 5.29	0 to 6 mo: 712
				7 to 12 mo: 8.36	7 to 12 mo: 987
				1 to 3 years: 2.59	1 to 3 years: 200
				EDI from IF only	
				0 to 6 mo: 2.91	0 to 6 mo: 536
				7 to 12 mo: 2.63	7 to 12 mo: 296
				1 to 3 years: 1.41[4]	1 to 3 years: 117[4]

Abbreviations: bw, body weight; EDI, estimated dietary intake; IbR, incorporated by reference; IF, infant formula; mo, months; NR, not reported.

[1] GRAS notices for 2'-FL which were withdrawn by the notifier, or for which FDA's response is still pending, are not listed in this table.

[2] The maximum intended use levels are listed here.

[3] Reflects cumulative exposure from both intended and current uses of 2'-FL in foods, excluding dairy-based foods, that may be consumed by infants and toddlers consuming hypoallergenic formula.

[4] Value may not be statistically reliable due to small sample size.

4. Self-Limiting Levels of Use

This Part is not applicable. The intended use of 2'-FL is not self-limiting.

5. Experience Based on Common Use in Food Before 1958

Although there is a history of safe consumption for 2'-FL by infants, including preterm infants, from its presence in human milk, the statutory basis for the conclusion of GRAS status for the intended use of 2'-FL in exempt infant formula for preterm infants (specifically post-discharge) is based on scientific procedures, and not common use in food before 1958.

6. Safety Narrative

6.1 Introduction

Breastmilk is widely recognized as the optimal form of nutrition for all infants, including preterm infants (Arslanoglu et al., 2019; Eidelman & Schanler, 2012; Koletzko et al., 2014; Lapillonne et al., 2019). "Preterm" is commonly defined as infants who are born at <37 weeks gestational age (Stewart & Barfield, 2019). Terminologies have been adopted to further subcategorize preterm infants according to their gestational age, including "extremely preterm" (less than 28 weeks), "very preterm" (28 to 32 weeks), "moderately preterm" (32 to 34 weeks), and "late preterm" (34 to 37 weeks) (Lapillonne et al., 2019; WHO, 2018). In the U.S., approximately 10% of all live births are preterm (Stewart & Barfield, 2019). Late preterm infants account for approximately 70% of these preterm births, while the other 3 subcategories each represent approximately 10% (Stewart & Barfield, 2019).

Preterm infants, especially those who are extremely preterm and very preterm, may accumulate significant energy, protein, mineral or other nutrient deficits during their initial hospital stay and they may be growth-restricted at discharge relative to gestational age-matched term infants (Aggett et al., 2006; Tudehope et al., 2013; Young et al., 2016). In a ESPGHAN position paper on the post-discharge feeding of preterm infants (Aggett et al., 2006), it was recommended that infants discharged home with a normal weight for post-conceptional age are not at increased risk of long-term growth failure, and could be fed similarly to term infants of similar gestational age, being breastfed when possible. On the other hand, infants discharged with a subnormal weight for postconceptional age are at increased risk of suboptimal growth. Breastfeeding or fortified human milk should be promoted, and if formula-fed, infants should receive a special nutrient-enriched post-discharge formula (Aggett et al., 2006). In a more recent ESPGHAN position paper on the feeding of late and moderately preterm infants specifically, human milk continues to be strongly endorsed as the preferred method of feeding (Lapillonne et al., 2019). The American Academy of Family Physicians also recommends that nutrient fortification of breast milk or enriched formula should be considered in premature infants who are less than the 10th percentile in weight for corrected age (Gauer et al., 2014).

Preterm post-discharge formulas are considered intermediary products with nutritional compositions that are between those of preterm formula typically given in hospitals, and standard formulas available for term infants. They are generally enriched in energy (72 to 74 kcal/100 mL) and protein (1.8 to 2.08 g/100 mL) when compared to a standard term infant formula, which has a typical energy content of 66 to 68 kcal/100 mL and protein concentrations of approximately 1.4 to 1.7 g/100 mL. Preterm post-discharge formulas may also be enriched with vitamins, minerals, and trace elements when compared to a standard term infant formula. While the macro- and micronutrient content of formula products can be readily set to match typical concentrations in breastmilk, and adjusted accordingly to meet the additional nutritional demands of a preterm infants, there are other unique components in breastmilk that are not present in commercial preterm post-discharge formulas. One notable compositional difference is that formula products, which are largely cow milk-based, do not contain the fraction of structurally diverse HMOs that are present in breastmilk (Bode, 2012). As discussed in Section 3.2.1 above, 2'-FL is one of the most abundant HMOs in the majority of mothers' breastmilk (Castanys-Muñoz et al., 2013; Coppa et al., 2004; Soyyılmaz et al., 2021). Manufactured 2'-FL preparations already have GRAS status for use in non-exempt term infant formula, at levels ranging from 2.0 to 2.4 g/L, as consumed (GRN Nos. 546, 571, 650, 735, 749, 852, 859, 897, 924, 932, 987). Chr. Hansen's 2'-FL is also GRAS for use in exempt hypoallergenic formula for term infants and in hypoallergenic toddler formula at up to 2.0 g/L, as consumed (GRN No. 929).

Considering that breastfeeding is strongly endorsed as the preferred method of feeding across all infant groups, there is a history of safe consumption of 2'-FL by both term and preterm infants alike. As described in Section 3.0, the intended use level of 2'-FL in preterm post-discharge formula (2.0 g/L, as consumed) is comparable to the concentration of 2'-FL reported in breastmilk, and accordingly, is expected to result in similar levels of intakes as those ingested by preterm infants who are fed breastmilk post-discharge. In addition to the history of safe consumption, the safety of 2'-FL has been demonstrated by an extensive dataset of preclinical toxicology studies and human clinical studies. These studies have been described in detail in previous GRAS notices for 2'-FL (GRN Nos. 546, 571, 650, 735, 749, 852, 859, 897, 924, 929, 932, 987), and are incorporated by reference in the sections below. To identify other publications pertinent to the evaluation of the intended uses of 2'-FL that have been published since these previous GRAS notices, a literature search was conducted up to October 2021.

The intended uses of 2'-FL in preterm post-discharge formula can be considered comparable to its existing uses in term infant formula. In addition to the numerous clinical studies conducted with 2'-FL in infant formula, recent clinical data indicate that 2'-FL supplementation is safe and supported normal growth in preterm infants even within a hospital setting (27 to 33 weeks gestation with birth weight <1700 g) (see Section 6.5.1). Thus, formulas containing 2'-FL are expected to be tolerated by stable preterm infants who have been discharged from the hospital, similar to healthy term infants.

6.2 Safety of the Production Strain

The safety of the host organism, *E. coli* BL21(DE3), is thoroughly summarized in GRN No. 571, which received a "no questions" letter from the U.S. FDA. In brief, *E. 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.

Given the widespread use of *E. coli* BL21(DE3) in various biotechnology applications, its use as the host strain for the manufacture of Chr. Hansen's 2'-FL are not expected to pose any safety concerns. It should also be noted that the 2'-FL production strain (*JBT-2FLΔlacZ*) was engineered with genes with known function, which do not confer toxicogenicity or virulence. Thus, *JBT-2FLΔlacZ* is non-toxigenic, not capable of DNA transfer to other organisms, and has the same virulence profile as *E. coli* BL21(DE3). Additionally, as described in Section 2.2, the production organism is removed through a series of purification steps employed during the manufacturing process of 2'-FL.

6.3 Absorption, Distribution, Metabolism, Excretion (ADME)

6.3.1 HMOs as Non-Digestible Carbohydrates

The ADME of HMOs has been extensively summarized in previous GRAS notices for 2'-FL (GRN Nos. 546, 571, 650, 735, 749, 852, 859, 897, 924, 929, 932, 987), and evaluations for 2'-FL published by worldwide authoritative bodies, such as EFSA (EFSA NDA Panel, 2015, 2019), and Food Standards Australia New Zealand (FSANZ) (FSANZ, 2021).

It is well established that HMOs, including 2'-FL, are recognized as non-digestible carbohydrates that are highly resistant to digestive enzymes and do not undergo any significant digestion in the upper gastrointestinal tract. *In vitro* studies have shown that HMOs are minimally digested when incubated with digestive enzyme preparations or intestinal brush border membranes (Engfer et al., 2000; Gnoth et al., 2000). *In vitro* experiments have also mechanistically examined whether HMOs are capable of crossing the epithelium of the small intestines. Using Caco-2 human intestinal epithelial cells, it has been suggested that neutral HMOs can be transported across the intestinal epithelium by receptor-mediated transcytosis as well as by paracellular transport, whereas acidic HMOs are absorbed *via* the non-specific paracellular transport only (Gnoth et al., 2001). Nonetheless, *in vivo* studies among infants and in rodents have reported that 1 to 2% of the total amount of ingested HMO is excreted unchanged in the urine, and that unabsorbed oligosaccharides pass through the gastrointestinal tract where it is either fermented by the resident microbiota or excreted unchanged in the feces (Brand-Miller et al., 1998; Chaturvedi et al., 2001; Coppa et al., 2001; Dotz et al., 2014; Goehring et al., 2014; Kuntz et al., 2019; Marriage et al., 2015; Obermeier et al., 1999; Rudloff et al., 1996, 2012; Ruhaak et al., 2014; Vazquez et al., 2017). Similar to term infants, detection of HMOs in urine and fecal samples of preterm infants generally correlates with the presence of these compounds in the dietary source (breastmilk, formula) (Albrecht et al., 2011; De Leoz et al., 2013; Rudloff et al., 1996; Underwood et al., 2015).

6.3.2 Developmental Processes

Anatomical development of the gastrointestinal tract is largely complete by 20 weeks of gestation, with further functional and biochemical maturations taking place throughout the third trimester and beyond (EFSA Scientific Committee et al., 2017; Fanaro, 2013; Henderickx et al., 2019). One important development that needs to take place in preterm infants after birth is the maturation of the gastrointestinal barrier. It has been suggested that immaturity of the intestinal epithelial barrier, along with an underdeveloped immune system and altered gut microflora, increase the hospitalized preterm infant's susceptibility to conditions such as necrotizing enterocolitis and sepsis (Halpern & Denning, 2015; Henderickx et al., 2019; Van Belkum et al., 2020).

Although preterm infants may have a "leaky gut" at birth, intestinal permeability progressively decreases during the first weeks of life as the intestinal barrier develops (Beach et al., 1982; Ma et al., 2018; Rouwet et al., 2002; Saleem et al., 2017; Shulman et al., 1998; Taylor et al., 2009; Van Elburg et al., 2003; Weaver et al., 1984; Westerbeek et al., 2011). Preterm and term infants also share typical immune system development patterns, which differ at birth, but quickly converge after birth. In an analysis of term and preterm infant immune cell populations by mass cytometry and immunoassays, Olin and colleagues characterized immune system development (Olin et al., 2018). Preterm infant immune cell population changes during the first weeks of life begins the process to a shared trajectory of immune system changes with term infants. Plasma protein changes contributed to preterm and term immune system convergence, especially changes in leptin and IL-8, which converged during the first month of life. Preterm and term infant immune system development converged by 3 months of age as evidenced primarily by changes in preterm infant neutrophil and naïve CD4+ T cell frequencies. Moreover, Grier and colleagues observed that although T cell phenotype and function clustered separately in preterm versus term infants at birth, they converged at 40 weeks postmenstrual age and were fully overlapping by 12 months corrected age (Callahan et al., 2021; Grier et al., 2020). Thus, while it is known that preterm infants' immune systems are immature at birth, their immune system matures rapidly postnatally to be more like that of term infants.

The benefits of breastmilk for all infants, including preterm infants, have been well recognized. Human milk, including the HMO component, is believed to play an important role in infant development (Granger et al., 2021; Hill et al., 2021; Vizzari et al., 2021). It should be highlighted that the intended use level for 2'-FL in preterm post-discharge formula will provide comparable levels of 2'-FL intake as preterm infants consuming human milk, and that these formula products will be consumed by stable infants who have met the criteria for hospital discharge, such as the ability to take feedings by mouth, along with other indices of functional maturation and physiologic stability (Jefferies et al., 2014; Stewart & Barfield, 2019; Whyte et al., 2010; Ziegler, 2019). Clinical data have also suggested supplementation with 2'-FL, or other non-digestible carbohydrates such as GOS and FOS, are safe and well-tolerated by preterm infants (Section 6.5).

6.4 Preclinical Studies

6.4.1 Overview

A number of preclinical toxicology studies have been conducted with Chr. Hansen's 2'-FL, including the standard battery of mutagenicity/genotoxicity assays, as well as repeated-dose animal studies. These studies have been described in detail in the previous GRAS notices (GRN No. 571 and GRN No. 929), and they are incorporated by reference herein. Furthermore, purified preparations of 2'-FL produced by other manufacturers, either by microbial fermentation with a genetically modified strain of *E. coli* K12 or chemical/enzymatic synthesis, have been extensively evaluated in toxicological studies. These studies have also been described in previous GRAS notices, and their key results are summarized in Tables 6.4.1-1 and 6.4.1-2 below. No evidence of genotoxicity/mutagenicity were observed across these studies, and no adverse effects have been observed in multiple sub-chronic (90-day) oral toxicity studies conducted in rats, including neonatal rats starting from postnatal day 7. The no-observed-adverse-effect level (NOAEL) was concluded to range from 5.0 to ~7.5 g/kg bw/day.

For the risk assessment of food substances intended for consumption by infants, the physiological development of the gastrointestinal tract of neonatal piglets are considered to be more similar to those of humans, and thus may be a more appropriate model (Constable et al., 2017; EFSA Scientific Committee et al., 2017). Chr. Hansen's 2'-FL has been evaluated in 2 neonatal piglet studies, either on its own or as a mixture in combination with other HMOs (LNT, 3-FL, 3'-SL, 6'-SL) (Hanlon, 2020; Hanlon & Thorsrud, 2014). Given the pertinence of these studies in supporting the intended uses of 2'-FL in infants, they are described further in Section 6.4.2 below.

Table 6.4.1-1 Summary of Genotoxicity Assays Conducted with 2'-FL

Reference	Test Substance	Method of Manufacture	Manufacturer	Study Type	Conclusions	GRAS Notice[1]
(Coulet et al., 2014)	2'-FL	Chemical synthesis	Glycom A/S	Bacterial reverse mutation test (OECD-compliant)	Not mutagenic	546
				I*n vitro* mammalian cell gene mutation assay (OECD-compliant)	Not mutagenic	546
Unpublished data in GRN No. 571 (Appendix M1, M2)	2'-FL	Fermentation	Chr. Hansen[2]	Bacterial reverse mutation test (OECD)	Not mutagenic	571
				In vivo micronucleus test in rats (OECD)	Not genotoxic	571
Unpublished (Verspeek-Rip, 2015)	2'-FL	Fermentation	Glycom A/S	Bacterial reverse mutation test (OECD)	Not mutagenic	650
Unpublished (Verbaan, 2015a)	2'-FL	Chemical synthesis	Glycom A/S	*In vitro* micronucleus test (OECD)	Not clastogenic or aneugenic	650
Unpublished (Verbaan, 2015b)	2'-FL	Fermentation	Glycom A/S	*In vitro* micronucleus test (OECD)	Not clastogenic or aneugenic	650

Reference	Test Substance	Method of Manufacture	Manufacturer	Study Type	Conclusions	GRAS Notice[1]
(Van Berlo et al., 2018)	2'-FL	Fermentation	Friesland Campina Domo	Bacterial reverse mutation test (OECD)	Not mutagenic	735
				In vitro micronucleus test (OECD)	Not clastogenic or aneugenic	735
(Phipps et al., 2018)	2'-FL and DFL mixture	Fermentation	Glycom A/S	Bacterial reverse mutation test (OECD)	Not mutagenic	815
				In vitro micronucleus test (OECD)	Not mutagenic	815
(Parschat et al., 2020)	2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL mixture	Fermentation	Chr. Hansen[2]	Bacterial reverse mutation test (OECD)	Not mutagenic	921
				In vitro micronucleus test (OECD)	Not clastogenic or aneugenic	921
Unpublished (Case and Yoon, 2020)	2'-FL	Fermentation	Advanced Protein Technologies Corp.	Bacterial reverse mutation test	Not mutagenic	932
				In vitro chromosome aberration test	Not clastogenic or aneugenic	
				In vivo micronucleus test in mice	Not genotoxic	

Abbreviations: 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose; DFL, difucosyllactose; LNT, lacto-*N*-tetraose.

[1] The GRAS notice in which the study was first described is listed here.

[2] Previously known as Jennewein Biotechnology, GmBH.

Table 6.4.1-2 Animal Toxicity Studies Conducted with 2'-FL

Reference	Test Substance	Method of Manufacture	Manufacturer	Study Type	NOAEL	GRAS Notice[1]
Rodent Studies						
(Coulet et al., 2014)	2'-FL	Chemical synthesis	Glycom A/S	14-day DRF study in rats	5 g/kg bw/day	546
				90-day oral toxicity study in neonatal rats (adapted OECD method)		
Unpublished data in GRN No. 571 (Appendix M3)	2'-FL	Fermentation	Chr. Hansen[2]	7-day pilot tolerance study in rats	7.6 g/kg bw/day (males); 8.72 g/kg bw/day (females)	571
				90-day dietary toxicity study in rats (OECD-compliant)		
Unpublished (Penard, 2015)	2'-FL	Fermentation	Glycom A/S	90-day oral toxicity study in neonatal rats (adapted OECD method)	5 g/kg bw/day	650
(Van Berlo et al., 2018)	2'-FL	Fermentation	Friesland Campina Domo	90-day dietary toxicity study in rats (OECD-compliant)	7.25 g/kg bw/day (males); 7.76 g/kg bw/day (females)	735
Unpublished (Flaxmer, 2017)	2'-FL and DFL mixture	Fermentation	Glycom A/S	14-day DRF study in rats	5 g/kg bw/day	815
(Phipps et al., 2018)	2'-FL and DFL mixture	Fermentation	Glycom A/S	90-day oral toxicity study in neonatal rats (adapted OECD method)	5 g/kg bw/day	

Reference	Test Substance	Method of Manufacture	Manufacturer	Study Type	NOAEL	GRAS Notice[1]
(Parschat et al., 2020)	2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL mixture	Fermentation	Chr. Hansen[2]	7-day pilot tolerance study in rats	5.67 g/kg bw/day (males); 6.97 g/kg bw/day (females)[3]	921
				90-day dietary toxicity study in rats (OECD-compliant)		
Unpublished (Case and Yoon, 2020)	2'-FL	Fermentation	Advanced Protein Technologies Corp.	Acute oral toxicity study in rats	LD_{50} >7.5 g/kg bw	932
				90-day oral toxicity study in rats (OECD-compliant)	7.5 g/kg bw/day	
Neonatal Piglet Tolerance Studies						
(Hanlon & Thorsrud, 2014)	2'-FL	Fermentation	Chr. Hansen[2]	21-day neonatal piglet tolerance study	2 g/L of 2'-FL in milk replacer (~0.29 g/kg bw/day)	571
(Hanlon, 2020)	2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL mixture	Fermentation	Chr. Hansen[2]	21-day neonatal piglet tolerance study	8 g/L of total HMOs in milk replacer (~3.6 g/kg bw/day)[4]	921

Abbreviations: 2'-FL, 2'-fucosyllactose; 3-FL, 3-fucosyllactose; 3'-SL, 3'-sialyllactose; 6'-SL, 6'-sialyllactose; bw = body weight; DFL, difucosyllactose; DRF, dose-range finding study; HMOs, human milk oligosaccharides; LD_{50}, median lethal dose; LNT, lacto-*N*-tetraose; NOAEL = no-observed-adverse-effect level.

[1] The GRAS notice in which the study was first described is listed here.

[2] Previously known as Jennewein Biotechnology, GmBH.

[3] The HMO mixture used in this study contained 47.1% 2'-FL by dry weight. Therefore, the NOAEL corresponds to 2'-FL intakes of 2.67 g/kg bw/day in males and 3.28 g/kg bw/day in females.

[4] The HMO mixture used in this study contained 49.1% 2'-FL by dry weight. Therefore, the HMO mixture provided ~3.9 g/L of 2'-FL, and the NOAEL corresponds to 2'-FL intakes of approximately 1.8 g/kg bw/day in males and females.

6.4.2 Tolerance Studies in Neonatal Piglets

6.4.2.1 Administration of 2'-FL (Hanlon & Thorsrud, 2014)

Details of this study have been presented in GRN No. 571 (pg. 31 and 32) and are incorporated by reference herein. In brief, a total of 27 male and 21 female Yorkshire piglets were administered a standard milk replacer (ProNurse® Specialty Milk Replacer), or the same milk replacer supplemented with 2'-FL at 200 mg, 500 mg or 2000 mg/L, starting from 2 days after birth for 21 days (Hanlon & Thorsrud, 2014).

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 2,000 mg/L group and in one female in the 500 mg/L dose group. The one male in the 2,000 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.

These results indicate that daily dietary administration of 2'-FL to neonatal piglets for 3 weeks following birth, at concentrations up to 2,000 mg/L in milk replacer, was well tolerated and did not produce any adverse treatment-related effects on growth and development. The intake of 2'-FL was calculated to be 291.74 and 298.99 mg/kg bw/day in males and females, respectively.

6.4.2.2 Administration of 2'-FL with other HMOs (Hanlon, 2020)

Details of this study have been presented in GRN No. 921 (pg. 38 to 70) and are incorporated by reference herein. In brief, a mixture of HMOs containing 2'-FL, 3'-FL, LNT, 3'-SL, and 6'-SL was administered to 2-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 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.

There were no treatment-related differences in body weight, food consumption, or feed efficiency between groups. Furthermore, there were no differences in hematology, clinical chemistry, or urinalysis parameters on Study Day 7 and Study Day 21 that could be attributed to HMO MIX 1, nor were there any findings in organ weights, or macroscopic and microscopic inspection of tissues that could be attributed to HMO MIX 1. 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. 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 related to the administration of HMO MIX 1, but rather due to an underlying bacterial infection that was likely obtained at the farm prior to enrollment in the study.

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 in milk replacer (providing 3.9 g/L of 2'-FL), was well-tolerated, did not produce adverse effects on growth and development. This dosage corresponds to calculated intakes of the HMO MIX 1 at 3.6 and 3.7 g/kg bw/day in males and females, respectively. Considering the HMO MIX 1 test article contained 49.1% of 2'-FL by dry weight, this corresponds to 2'-FL intakes of approximately 1.8 g/kg bw/day in males and females.

6.5 Clinical Studies

6.5.1 Studies Conducted with 2'-FL in Infants

Considering breastfeeding is strongly endorsed as the optimal form of nutrition for all infants, there is a history of safe consumption of 2'-FL by both term and preterm infants. It has also been demonstrated that formulas containing 2'-FL are safe and well-tolerated by infants in a number of clinical studies, including one study that involved preterm infants within a hospital setting (Hascoët et al., 2021). The studies that have been described extensively in other previous GRAS notices for 2'-FL (GRN Nos. 650, 735, 749, 815, 852, 897, 929) are incorporated by reference herein, and their summary is available in Table 6.5.1-1 (Berger et al., 2020; Goehring et al., 2016; Kajzer et al., 2016; Marriage et al., 2015; Nowak-Wegrzyn et al., 2019; Puccio et al., 2017; Storm et al., 2019). For more recent studies that have been published since the filing of other GRAS notices for 2'-FL (Hascoët et al., 2021; Parschat et al., 2021; Ramirez-Farias et al., 2021; Riechmann et al., 2020; Vandenplas et al., 2020), a description is provided below along with a tabular summary in Table 6.5.1-1.

Infant Clinical Studies Not Described in Previous GRAS Notices for 2'-FL

A. 2'-FL in Preterm Formula

The results of a randomized, double-blind, placebo-controlled trial evaluating the effects of 2'-FL supplementation in preterm infants were recently presented at the 6th World Congress of Pediatric Gastroenterology, Hepatology and Nutrition held June 2 to 5, 2021 (Hascoët et al., 2021). For this study, preterm infants at 27 to 33 weeks gestation with birth weight <1700 g were randomized as soon as possible after birth from seven different neonatal units in France. The infants (n=43/group) received either a supplement providing 374 mg/kg bw/day of 2'-FL and lacto-*N*-neotetraose (LNnT) in a 10:1 ratio (corresponding to 340 mg/kg bw/day of 2'-FL and 34 mg/kg bw/day of LNnT), which was termed the "HMO group", or an isocaloric placebo consisting of only glucose (140 mg/kg bw/day) until discharged from the neonatal unit.

The mean chronological age at the initiation of supplementation was 6.3 days (HMO group) and 6.2 days (placebo). Non-inferiority in the number of days to reach full enteral feeding from birth, which was the primary outcome of interest and is indicative of feeding tolerance, was achieved for the HMO group vs. placebo in the full analysis set, with similar results observed in the per protocol set. A non-significant trend towards improved feeding tolerance was observed in the HMO group, for which the adjusted mean time to reach full enteral feeding from birth was two days shorter when compared to placebo (12.2 days vs. 14.3 days). There was no significant difference in the weight-for-age z-scores between groups at any time point throughout the full enteral feeding period until discharge. Compared to placebo, the HMO group had significantly higher length-for-age z-scores at full enteral feeding Day 14 ($p = 0.037$) and Day 21 ($p = 0.037$), and significantly higher head circumference-for-age z-scores at discharge ($p = 0.07$). These results suggest supplementation with 2'-FL (and LNnT) support early postnatal growth, which was in line with the desired growth velocity of preterm infants. Measures of gastrointestinal tolerance, including daily gastric residuals, stool frequency and consistence, and incidence of gastrointestinal adverse events, were similar between the HMO and placebo groups. The incidence of necrotizing colitis was low in both groups. The incidence of other illnesses and infections were comparable between the HMO (n = 22 [50%])

and placebo (n = 18 [42.9%]) groups. It was concluded that supplementation with 2'-FL and LNnT is safe and well tolerated in preterm infants.

B. 2'-FL at a Higher Use Level (3.0 g/L) than Previously Tested – NCT04105686

Abbott Nutrition completed a growth monitoring study (ClinicalTrials.gov identifier: NCT04105686), which compared the growth of infants receiving a milk-based experimental formula that contained a mixture of five commercially prepared HMOs (3.0 g/L 2'-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3'-SL and 0.28 g/L 6'-SL) to the growth of infants receiving the same formula without HMOs (control). A human milk-fed reference group (HM) was also included. The study was a 16-week randomized, controlled, blinded growth and tolerance study. Healthy term infants (n=366) were enrolled in the study between birth and 14 days of age.

The primary variable of the study was weight gain per day from 14 to 119 days of age of infants in the two formula groups. Values at days 14, 38, 42, 56, 84 and 119 of life were used for the primary analysis. Results comparing the two infant formula groups to each other and to a human milk reference group for weight gain per day from 14 to 119 days of age indicated that there were no statistically significant differences in growth. Sensitivity analysis likewise showed no statistically significant differences among the three groups. Furthermore, the experimental formula was non-inferior to control using a non-inferiority margin of 3 g/day in primary and sensitivity analyses. Both formulas were well tolerated. In conclusion, this clinical study demonstrated that a formula containing up to 3.0 g/L of 2'-FL was safe, well tolerated and supported normal growth by infants.

C. 2'-FL at a Higher Use Level (3.0 g/L) than Previously Tested – NCT03513744

A multi-centered, randomized, double-blinded, controlled, parallel group clinical study was conducted to evaluate the safety and tolerability of a mixture of five commercially prepared HMOs (2.99 g/L 2'-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3'-SL and 0.28 g/L 6'-SL) (ClinicalTrials.gov identifier: NCT03513744). The results of this study have been published by Parschat et al. (2021).

Healthy term infants ≤14 days of age were randomized to receive exclusive feeding with an infant formula containing 5HMO-MIX (n=113), a control infant formula (n=112), or exclusive feeding with breastmilk as a reference control (n=116), for 4 months. The formula supplemented with HMOs was considered non-inferior to the control formula with respect to mean daily body weight gain. There were no differences in weight, length or head circumference gain between the two formula groups. The formula containing the HMOs was well tolerated, and the occurrence of adverse events was similar across all groups. Infants receiving formula containing HMOs and breastmilk produced slightly softer stools at a higher stool frequency than the control formula group. The study authors concluded that infant formula containing a mixture of HMOs, including 3.0 g/L of 2'-FL, is safe and well-tolerated by infants during the first months of life.

D. 2'-FL in Extensively Hydrolyzed Formula

A multi-center, open-label, single-arm study was conducted to evaluate the growth, tolerance, and compliance of an extensively hydrolyzed formula supplemented with 2'-FL (Ramirez-Farias et al., 2021). Infants less than 60 days of age with a suspected food protein allergy, persistent feeding intolerance, or presenting conditions where an extensively hydrolyzed formula was deemed appropriate, were enrolled

(n=48). The infants in this study had already been consuming extensively hydrolyzed formula (without HMOs) and were switched to receive a hypoallergenic casein-based extensively hydrolyzed formula with 0.2 g/L of 2'-FL as their sole source of nutrition for 2 months.

One infant never received the test formula, while 11 infants failed to meet one or more evaluability criteria, including consumption of non-study feeding for more than 5 days (n = 2), use of medications that may affect gastrointestinal tolerance (n = 1), anthropometric measurement at Day 60 obtained outside the window (n = 1), premature discontinuation of study product (n = 6) and lost to follow-up (n = 1). The test formula supported appropriate growth, with statistically significant improvement in weight-for-age z-scores from Day 1 to Day 60. After 60 days on the test formula with 2'-FL, persisting symptoms (diarrhea, constipation, blood in stool, vomiting, spit-up/gagging/reflux, fussiness, rash or eczema) either remained the same, improved, or resolved when compared to baseline. Adverse events were observed in 15 infants in the study, with most AEs being mild in severity and deemed by the investigators as not related to product. The most common reported AEs were seborrheic dermatitis (five infants), gastrointestinal reflux (three infants), and infantile spit-up (2 infants). The test formula was considered safe and well tolerated.

E. 2'-FL with LNnT in Partially Hydrolyzed Formula (Open-Label)

An open-label, prospective study was conducted to evaluate the growth and tolerability of an infant formula containing HMOs (2'-FL and LNnT) (Riechmann et al., 2020). Healthy term infants were enrolled at age 7 days to 2 months. The study included 3 groups: exclusively formula-fed infants consuming a partially hydrolyzed 100% whey formula with 1.0 g/L of 2'-FL and 0.5 g/L of LNnT (along with *Lactobacillus reuteri* (DSM 17938)) (n=82); infants mixed-fed infant formula and human milk (n=62); and exclusively breastfed infants as a reference control (n=63). The formula-fed and mixed-fed infants received the test formula for approximately 8 weeks.

There were no significant differences in anthropometric measures between groups, with age appropriate growth observed in all groups. The incidence of adverse events was generally low and not significantly different among the groups. Three infants experienced potentially product-related adverse events, with 2 incidences of cow-milk intolerance (1 in formula-fed and 1 in mixed-fed groups), and 1 instance of irritability in the formula-fed group. Six serious adverse events occurred (bronchiolitis) but were not considered related to the study feeding. 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- week timepoints.

F. 2'-FL with 3'-GL, GOS, and lcFOS

A multi-site, double-blind, randomized, controlled study was conducted in healthy term infants to evaluate the growth, safety, and tolerance of a novel formula (Vandenplas et al., 2020). A total of 215 fully formula-fed infants ≤14 days of age were randomized to receive a nutritionally complete cow milk-based test formula (n=108) or a control (n=107) formula until 17 weeks of age. The test formula contained 1.0 g/L of 2'-FL; 0.15 g/L of 3'-galactosyllactose (3'-GL), which is a HMO identified in fermented infant formula as a by-product of the Lactofidus fermentation process; 8 g/L of a GOS/lcFOS mixture (9:1 ratio), and anhydrous milk fat (49.8% of total fat). The control formula was a commercially available

standard infant formula containing GOS/lcFOS (0.8 g/100 mL; 9:1), but no 2'-FL, 3'-GL, or milk fat. A group of breastfed infants (n = 61) was also included as a reference control.

The dropout rate was similar between the test (16%) and control (17%) formula groups. Growth parameters (gains in body weight, length, and head circumference) were demonstrated to be equivalent between the test and control formula groups. The estimated z-scores for weight-for-age, length-for-age, BMI-for-age, and head circumference-for-age were all within ±1 SD of WHO growth standards for formula groups and breastfed reference group, indicative of adequate infant growth. There were no statistically significant differences in the number of total or specific adverse events, or in the number of serious adverse events, between the test and control formula groups. The incidence of frequent regurgitation and vomiting were comparable between the test and control formula groups, and the distribution of infants across the different ratings of the stool consistency scores (watery, soft, formed, hard) were not significantly different across all groups. The study authors concluded the novel formula supports adequate infant growth and is safe and well-tolerated in healthy term infants.

Table 6.5.1-1 Clinical Studies Conducted with 2'-FL in Infants

Reference	Study Design	Study Population	Interventions (# of Infants at Randomization)	Duration of Intervention	Main Outcomes
Preterm Formula					
(Hascoët et al., 2021)* -- conference abstract	Multi-center, randomized, double-blind, controlled, parallel study	Preterm infants Birth weight <1700 g GA: 27 to 33 weeks	**Test**: Supplement with 2'-FL and LNnT (10:1 ratio) at 374 mg/kg bw/day (n=43) **Control**: Isocaloric placebo supplement containing only glucose (140 mg/kg bw/day) (n=43)	As soon as possible after birth until discharged from the neonatal unit	• Non-inferiority in time to reach full enteral feeding in test group vs. control (full analysis set), with similar results in per protocol set. • Adjusted mean time to reach full enteral feeding was 2 days shorter in test group (12.2 days) vs. control (14.3 days), though difference is NSD. • NSD in weight-for-age z-scores between groups from full enteral feeding to discharge. Compared to controls, test group had SS ↑ length-for-age z-scores at full enteral feeding Day 14 and Day 21, and SS ↑ head circumference-for-age z-scores at discharge. • Measures of gastrointestinal tolerance, including daily gastric residuals, stool frequency and consistence, and incidence of gastrointestinal adverse events, were similar between the HMO and placebo groups. • The incidence of necrotizing colitis was low in both groups. • The incidence of other illnesses and infections were comparable between the HMO (n = 22 [50%]) and placebo (n = 18 [42.9%]) groups. • It was concluded that supplementation with 2'-FL and LNnT is safe and well tolerated in preterm infants.
Standard Infant Formulas					
Abbott Nutrition (unpublished data)*	Randomized, blinded, controlled, parallel study NCT04105686	Healthy term infants 0 to 14 days of age (n=366)	**Test**: Formula with 2.99 g/L 2'-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3'-SL and 0.28 g/L 6'-SL **Control**: Formula with no HMOs **HM Reference**: Infants fed human milk	4 months	• NSD in body weight gain between test and control groups. Test formula was non-inferior to the control formula with respect to growth. • Test formula was considered well-tolerated. • Test formula containing HMO mixture was concluded to be safe, well-tolerated, and supported normal growth by healthy term infants.

Reference	Study Design	Study Population	Interventions (# of Infants at Randomization)	Duration of Intervention	Main Outcomes
(Marriage et al., 2015) -- safety & tolerance (Goehring et al., 2016) -- sub-analysis on immune parameters	Multi-center, randomized, double-blind, controlled, parallel study NCT01808105	Healthy term infants 0 to 5 days of age	**EF1**: Formula with 2.2 g/L GOS + 0.2 g/L 2'-FL (n=104) **EF2**: Formula with 1.4 g/L GOS + 1.0 g/L 2'-FL (n=109) **Control**: Formula with 2.4 g/L GOS (n=101) **HM Reference**: Breastfed infants (n=106)	Until 119 days of age	Marriage et al., 2015: • NSD in the number of non-completers among the formula-fed groups. • NSD (sex-specific or sex- combined) in mean weight, length, or head circumference among feeding groups during the study, and NSD among feeding groups in mean gains in these measures from day 14 to 119. • The mean number of stools/day was SS ↑ for the HM group compared to all formula groups in the 3-day period before the study visits at day 28, 42, and 84. The mean number of stools/day was also SS ↑ for the HM group compared to control formula in the 3-day period before the day 119 visit. • NSD in mean rank stool consistency score between formula groups. • Spitting-up or vomiting was SS↑ in the formula-fed groups compared to the HM group from enrollment to day 28, though there was NSD after day 28. • NSD in the overall percentage of subjects experiencing AEs or serious AEs in the formula-treated groups. The control formula and EF2 had SS ↑ infants with AEs in the "infections and infestations" category compared to EF1, but the types of AEs were similar (upper respiratory tract symptoms; otitis media, viral infections, and oral candidiasis). • The study authors concluded formula supplemented with 2'-FL is safe, well tolerated, and supports growth patterns similar to HM-fed infants. Goehring et al., 2016 • Blood samples were analyzed from a subset of the participants in the control formula (n=39), EF1 (n=37), EF2 (n=37) and HM (n=42) groups • Study authors concluded that formula containing 2'-FL modified innate and adaptive immune profiles to be more like that of breastfed infants.
(Parschat et al., 2021)*	Multi-center, randomized, double-blind, controlled, parallel study NCT03513744	Healthy term infants ≤14 days of age	**Test**: Formula with 2.99 g/L 2'-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3'-SL and 0.28 g/L 6'-SL (n=113) **Control**: Formula with no HMOs (n=112)	4 months	• NSD in body weight, length or head circumference gain between test and control formula groups. Test formula was non-inferior to the control formula with respect to growth (i.e., body weight gain). • Test formula was considered well-tolerated. • Infants in test formula group, and the breastfed infants, had slightly softer stools at higher stool frequency. • Occurrence of AEs was similar across all groups. • Test formula containing HMO mixture was concluded to be safe, well-tolerated, and supported normal growth by healthy term infants.

Reference	Study Design	Study Population	Interventions (# of Infants at Randomization)	Duration of Intervention	Main Outcomes
			HM Reference: Exclusively breastfed infants (n=116)		
(Puccio et al., 2017) -- safety & tolerance (Berger et al., 2020; Dogra et al., 2021) -- microbiota analysis	Multi-center, randomized, double-blind, controlled, parallel study NCT01715246	Healthy term infants 0 to 14 days old	**Test**: Formula with 1.0 g/L 2'-FL and 0.5 g/L LNnT (n=88) **Control**: Formula with no HMOs (n=87) **HM Reference**: Exclusively breastfed infants for first 4 months included as control for microbiome analysis (n=38)	Exclusive formula feeding for 4 months, after which complementary foods were introduced. At 6 months of age, all infants were switched to a non-HMO containing follow-up formula until 12 months of age.	• The dropout rate was comparable between groups (n=20 in control; n=24 in test). The most common reason for discontinuation was an AE (n=11 in control; n=12 in test). • At 3 months, the stool microbiota profile in test formula group appeared closer to that of breastfed infants than those in the control formula group (Berger et al., 2020). • NSD in mean weight, length, head circumference, and BMI between groups. NSD in weight gain, mean weight-for-age, length-for-age, head circumference-for-age, and BMI-for-age z scores between groups. • NSD in GI symptoms, including flatulence, spitting-up and vomiting, between groups. NSD in parental-reported AEs between groups. • Parent-reported infant behavioral patterns including restlessness/irritability and colic were similar in the test 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 test 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. • Infant formula supplemented with 2'-FL and LNnT is safe, well-tolerated and supports age-appropriate growth.
(Kajzer et al., 2016) -- conference abstract Details also available in a review by	Multi-center, randomized, double-blind, controlled, parallel study	Healthy term infants 0 and 8 days of age	**Test**: Formula with 0.2 g/L 2'-FL and 2 g/L scFOS (n=46) **Control**: Formula with no oligosaccharides (n=42)	Until 35 days of age	• Thirty-six (86%) infants in the group receiving test formula, 41 (89%) in the control formula, and 42 (98%) in the HM group completed the study. • NSD in stool consistency, average volume of study formula intake, number of study formula feedings/day, anthropometric data, or percent feedings with spit-up/vomit among the groups. • The average number of stools per day for the HM group was SS ↑ in the HM group than both formula-fed groups. • An experimental formula containing 2'-FL and scFOS was safe and well tolerated.

Reference	Study Design	Study Population	Interventions (# of Infants at Randomization)	Duration of Intervention	Main Outcomes
Reverri et al., 2018			**HM Reference**: Exclusively fed human milk (n=43)		
(Vandenplas et al., 2020)*	Multi-center, randomized, double-blind, controlled, parallel study NCT03476889	Healthy term infants 0 to 14 days old	**Test**: Formula with 1.0 g/L 2'-FL, 8 g/L of a GOS/lcFOS mixture (9:1 ratio), 26% fermented formula providing 0.15 g/L of 3'-GL, and anhydrous milk fat (49.8% of total fat) (n=108) **Control**: Formula with 8 g/L of a GOS/lcFOS mixture (9:1 ratio), but no 2'-FL, 3'-GL, or milk fat (n=107) **HM Reference**: Fully breastfed infants consuming mother's own milk (n=61)	Until 17 weeks of age	• The dropout rate was similar between the test (16%) and control (17%) formula groups. • Growth parameters (total and daily gains in body weight, length, and head circumference) were equivalent between the test and control formulas. • Weight-for-age, length-for-age, BMI-for-age, and head circumference-for-age z-scores were within WHO growth standards for all formula and HM groups, indicative of adequate infant growth. • NSD in numbers of total or specific AEs, or in number of serious AEs, between test and control formula. The most common AEs were GI-related, occurring in 20.6% of infants in the test group, 16.3% in the control group, and 9.8% in the HM group. • Incidence of frequent regurgitation and vomiting were comparable between the test and control formula groups. • NSD in stool consistency scores between groups. • The study authors concluded the novel formula supports adequate infant growth and is safe and well-tolerated in healthy term infants.
Hydrolyzed Formulas					
(Nowak-Wegrzyn et al., 2019)	To evaluate whether EHF with HMOs meet hypo-allergenicity criteria using DBPCFC administered	Children age 2 months to 4 years with documented cow milk protein allergy (n=67)	**Test**: 100% whey EHF containing 1.0 g/L 2'-FL and 0.5 g/L LNnT **Control**: Commercially available whey-based EHF confirmed to be hypoallergenic	If DBPCFCs are negative, the participants completed a 1-week open-label food challenge with the test formula	• 64 children completed at least one DBPCFC, 62 children completed both, though 1 child was erroneously administered the test formula during both challenges. • 1 child reacted during the DPBCFC to both the test and control formula. Hypo-allergenicity criteria was considered met for both formulas since at least 90% of infants in the study tolerated it. • 61 children completed the 1-week open-label phase with the test formula. ○ One participant vomited on Day 1 of the home challenge but completed the home challenge without further problems.

Reference	Study Design	Study Population	Interventions (# of Infants at Randomization)	Duration of Intervention	Main Outcomes
	in a cross-over manner NCT03236207				○ One participant developed diarrhea on the last day of the challenge, which the site investigator attributed to gastroenteritis. ○ No significant GI symptoms (flatulence, abnormal stool frequency/ consistency, increased spitting-up, or vomiting) were reported. • No serious AEs occurred during the entire study.
(Ramirez-Farias et al., 2021)*	Multi-center, non-randomized, single-arm, study NCT03884309	Infants less than 60 days of age with conditions that warrant use of an EHF, such as persisting feeding intolerance, suspected food protein allergy sensitivity	**Test**: Hypoallergenic casein-based EHF with 0.2 g/L 2'-FL (n=48) Infants meeting eligibility criteria were switched from their current EHF to the test formula with 2'-FL.	60±5 days	• 1 infant never received the test formula. 11 infants failed one or more evaluability criteria, including consumption of non-study feeding for more than 5 days (n = 2), use of medications that may affect GI tolerance (n = 1), anthropometric measurement obtained outside the window at end-of-study (n = 1), premature discontinuation of study product (n = 6), and lost to follow-up (n = 1). • Reasons for premature discontinuation of formula were as follows: Parent reported AE (n = 1), Investigator reported AE (n = 1), Parent requested discontinuation for reason other than AE (n = 2), Non-compliance (n = 1) and Lost to follow-up (n = 1). • The test formula supported appropriate growth, with SS ↑ weight z-score from day 1 to day 60. • AEs were observed in 15 infants in the study, with most AEs being mild in severity and deemed by the investigators as not related to product. The most common reported AEs were seborrheic dermatitis (five infants), gastrointestinal reflux (3 infants), and infantile spit-up (2 infants). • After 60 days of consuming the test formula with 2'-FL, persisting symptoms (diarrhea, constipation, blood in stool, vomiting, spit-up/gagging/reflux, fussiness, rash or eczema) either remained the same, improved, or resolved when compared to baseline. • Study authors concluded the test formula was well tolerated, safe and supported growth in the intended population.
(Riechmann et al., 2020)*	Multi-center, non-randomized, open-label, study NCT04055363	Healthy term infants 7 days to 2 months old	**Test**: 100% whey partially hydrolyzed formula containing 1.0 g/L 2'-FL, 0.5 g/L LNnT, and *Lactobacillus reuteri* (n=82)	8 weeks	• Number of dropouts was similar between the exclusively formula-fed (n=16), mixed-fed (n=14) and HM (n=18) groups. • NSD in anthropometric measures between groups. Weight-for-age, length-for-age, and BMI-for-age z-scores were similar between groups, with mean z-scores within ±0.5 of the WHO medians at week 8. • Composite IGSQ scores demonstrated low gastrointestinal distress in all feeding groups, with NSD between groups at baseline, 4, or 8 weeks. NSD among the groups in the gassiness, fussiness, crying or spitting-up/vomiting domains of the IGSQ. For the stooling domain, exclusively

Reference	Study Design	Study Population	Interventions (# of Infants at Randomization)	Duration of Intervention	Main Outcomes
			Mixed-Fed: Infants consuming study formula and human milk (n=62) **HM Reference**: Exclusively fed human milk (n=63)		formula-fed infants had scores that were closer to the stooling profile of the HM group. • NSD in the incidence of AEs in test group (n=19), mixed-fed group (n=21), and HM group (n=18). Three infants experienced potentially product-related AEs, including two instances of cow milk intolerance (one each in exclusive formula and mixed-fed groups), and one instance of irritability in exclusive formula-fed group. • Six serious AEs occurred in the formula-fed (n=4) and mixed-fed (n=2) groups, all of which were bronchiolitis and considered unrelated to the study feeding by the investigators.
(Storm et al., 2019)	Multi-center, randomized, double-blind, controlled study NCT03307122	Healthy term infants 14±5 days old at enrollment	**Test**: 100% whey partially hydrolyzed formula containing *Bifidobacterium animalis* ssp. *lactis* Bb12 and 0.25 g/L of 2'-FL (n=39) **Control**: Same as test formula but without 2'-FL (n=40)	42 days	• Number of dropouts was similar between the test (n=9) and control (n=7). • Body weight and length, and weight-for-age and length-for-age, were similar between groups at the baseline and 6-week visit. • NSD in IGSQ scores between groups at baseline or end-of-study. • NSD in stool frequency and consistency between the groups over the course of the study. Significantly more stools were reported to be difficult to pass in the control than in the test group ($p<0.05$); however, the number of infants with stools reported as difficult to pass was NSD between groups. • Crying and fussing duration, vomiting frequency were similar between groups. NSD between groups in the proportion of infants reported to have any spit up over the 2-day diary period before the 6-week visit. Among the infants whose caregivers reported spit-up, significantly more were reported to have spit up >5 times/day in the 2'-FL group compared to controls. • There were no serious AEs in the study, and the frequency of AEs were equally distributed among the two groups. SS ↑ number of infants that experienced "infections and infestations" in the control group (n=9) than in the 2'-FL group (n=3) (p=0.05). • Study authors concluded the addition of 2'-FL to a partially hydrolyzed whey formula with *B. animalis* ssp. *lactis* Bb12 is safe and well-tolerated.

Abbreviations: 2'-FL, 2'-fucosyllactose; 3'-GL, 3'-galactosyllactose; AEs, adverse events; BMI, body mass index; DBPCFC, double-blind, placebo-controlled food challenges; EHF, extensively hydrolyzed formula; GA, gestational age; GI, gastrointestinal; GOS, galacto-oligosaccharides; HM, human milk; HMOs, human milk oligosaccharides; IGSQ, Infant Gastrointestinal Symptom Questionnaire; lcFOS, long-chain fructo-oligosaccharides; LNnT, lacto-*N*-neotetraose; NSD, no statistically significant difference; scFOS, short-chain fructo-oligosaccharides; SS, statistically significant.

[1] References denoted with an asterisk (*) have not been previously described in other GRAS notices.

6.5.2 Studies Conducted with 2'-FL in Older Children and Adults

A randomized, double-blind, controlled clinical study was recently published that evaluated the effects of a "young child formula" (YCF) supplementation on the incidence of gastrointestinal and upper respiratory infections among children age 1 to 2.5 years (Leung et al., 2020). The children (n=146) received 1 of 4 interventions for 6 months: a standard milk-based formula (YCF-ref); a milk formula containing 3 g/L of 2'-FL, immunoglobins (1 g/L), lactoferrin (1.7 g/L), TGF-beta (15 µg/L), and milk fat (2.5 g/100 mL) (termed YCF-A); a milk formula that is the same as YCF-A but with lower levels of immunoglobulins (0.1 g/L), lactoferrin (0.1 g/L), and no added 2'-FL or milk fat (termed YCF-B); or a milk formula that is the same as YCF-ref but with 3 g/L of 2'-FL (termed YCF-C). All 4 formulas also contained 4 g/L of GOS. The children consumed two 200 mL servings of the YCF daily (400 mL/day) for 6 months. No "remarkable between-group differences" were observed in anthropometric parameters, assessed as the z-scores for weight-for-age, height-for-age, and weight-for-height. The incidence of adverse events and serious adverse events were similar across groups, with no reported cases of product-related events as judged by investigators and confirmed by an independent data safety monitoring board. The study authors concluded all the YCFs tested were considered safe and supported normal growth.

Clinical studies have also evaluated the effects of 2'-FL supplementation in older children and in adults. Supplementation with 2'-FL, either alone or as a 4:1 mixture with LNnT, at 4.5 g/day for 8 weeks was concluded to be safe and well tolerated in overweight/obese children between the ages of 6 to 12 years old (Fonvig et al., 2021). In one randomized, placebo-controlled, double-blind, parallel study designed to assess safety and tolerability, ingestion of up to 20 g/day of either 2'-FL, LNnT, or a combination of 2'-FL and LNnT at a 2:1 ratio, was concluded to be well tolerated in healthy adults (Elison et al., 2016). Supplementation with 2'-FL was also reported to be well tolerated in adults with gastrointestinal conditions (e.g., irritable bowel syndrome) (Iribarren et al., 2020; Palsson et al., 2020; Ryan et al., 2021). These studies have limited relevance on the intended uses of 2'-FL in post-discharge formulas for preterm infants and therefore are not discussed further.

6.5.3 Studies Conducted with Other Non-Digestible Carbohydrates in Preterm Infants

A number of randomized controlled clinical trials have been conducted to investigate the effects of non-digestible oligosaccharides [*e.g.,* short-chain GOS (sc-GOS), long-chain FOS (lc-FOS), and pectin-derived acidic oligosaccharides (pAOS)] in preterm infants.

These studies have been examined in several systematic reviews (Chi et al., 2019; Mugambi et al., 2012; Srinivasjois et al., 2013). In the most recent review, a meta-analysis of 18 clinical trials of preterm infants (<2,500 g or <36 weeks) suggested that supplementation with non-digestible carbohydrates[3] had a significant decrease in the incidence of sepsis (risk ratio (RR): 0.64, 95% CI: 0.51, 0.78), mortality (RR: 0.58. 95% CI: 0.36, 0.94), length of hospital stay (mean difference (MD): −5.18, 95% CI: −8.94, −1.11), and time to full enteral feeding (MD: −0.99, 95% CI: −1.15, −0.83) (Chi et al., 2019). There were no significant differences in feeding intolerance (RR: 0.87, 95% CI: 0.52, 1.45) or morbidity rate of necrotizing

[3] The study authors included clinical trials that evaluated one of the following interventions: sc-GOS, lc-FOS, pAOS, oligosaccharides, fructans, inulin, or oligofructose.

enterocolitis (RR: 0.79, 95% CI: 0.44, 1.44). The study authors concluded that supplementation with non-digestible oligosaccharides, at levels as high as 1.5 g/kg bw/day is safe in preterm infants (Chi et al., 2019).

Thus, in addition to the history of safe consumption of HMOs through human milk, the lack of adverse effects from the administration of non-digestible carbohydrates in these studies further corroborates their safety in preterm infants.

6.6 Conclusion of GRAS Status

The safety of Chr. Hansen's 2'-FL as an ingredient for its intended use in preterm post-discharge formula is supported by the following:

- HMOs represents the third largest solid component of human milk, with 2'-FL being one of the most abundant oligosaccharides present.

- The GRAS status of Chr. Hansen's 2'-FL for use in non-exempt term infant formula (GRN No. 571) and in exempt hypoallergenic formula for term infants (GRN No. 929) at up to 2.0 g/L has been notified to the U.S. FDA and filed with "no questions".

- As detailed in those previous GRAS notices, 2'-FL manufactured by Chr. Hansen is chemically and structurally identical to 2'-FL in human milk. The production process is conducted in accordance with cGMP, and strict manufacturing controls are in place. The finished material is a spray-dried, powder containing ≥90% 2'-FL dry weight, with the remaining components comprising small amounts of residual carbohydrate by-products, ash, and moisture. The production organism, *E. coli* BL21(DE3) #1242, is safe for use; it is non-toxigenic and not capable of DNA transfer to other organisms. A series of purification steps are included in the manufacturing process to remove the production organism, and no residual DNA from the production strain remains in the finished 2'-FL material.

- Breastmilk is widely recognized as the optimal form of nutrition for all infants, including preterm infants. The intended use level of 2'-FL in preterm post-discharge formula (2.0 g/L) is within the ranges of 2'-FL concentrations that have been reported in human milk following preterm and term births. Accordingly, the estimated daily intakes of 2'-FL from its intended uses (up to 520 mg/kg bw/day) are considered comparable to those of post-discharged preterm infants who are fed human milk (up to 720 mg/kg bw/day).

- The safety of Chr. Hansen's 2'-FL has been demonstrated in preclinical toxicological studies, including mutagenicity/genotoxicity assays (bacterial reverse mutation assay, *in vivo* micronucleus test), and a 90-day oral toxicity study in rats. Toxicological studies (bacterial reverse mutation assay, *in vitro* micronucleus test, 90-day oral toxicity study) have also been conducted with Chr. Hansen's 2'-FL when tested as part of a mixture with other HMOs. Additionally, a number of preclinical studies have been conducted with 2'-FL preparations produced by other manufacturers. Generally, no adverse effects were observed in these studies, and the NOAEL was concluded to range from 5.0 to ~7.5 g/kg bw/day.

- Chr. Hansen has also conducted two separate 21-day tolerance studies in neonatal piglets, which are considered a suitable model of the physiological development of the infant gastrointestinal tract. These studies demonstrated that milk replacer containing 2'-FL at up to 2 g/L, or an HMO mixture at up to 8 g/L (providing 3.9 g/L of 2'-FL), was safe and well-tolerated.

- A number of clinical studies have further demonstrated the safety and tolerance of formulas supplemented with 2'-FL for term infants (at up to 3.0 g/L) and young children age 1 to 2.5 years (at 3.0 g/L). Consumption of 2'-FL at up to 20 g/day was also shown to be safe and well-tolerated by adults.

- HMOs, including 2'-FL, are largely resistant to the digestive enzymes in the upper gastrointestinal tract, with unabsorbed oligosaccharides being either fermented by the resident microbiota or excreted unchanged in the feces. Supplementation with 2'-FL at 340 mg/kg bw/day (with 34 mg/kg bw/day of LNnT) in preterm infants (27 to 33 weeks gestation, birth weight <1700 g) until hospital discharge was safe, well-tolerated, and supported normal growth. Clinical studies have also been conducted where other non-digestible carbohydrates (e.g., GOS, FOS) were administered to preterm infants without adverse effects. Together, these studies help to support that the intended use of 2'-FL in preterm post-discharge formula is similarly safe and well-tolerated, particularly given that 2'-FL has a history of safe consumption by these infants through its presence in breastmilk.

All pivotal data and information used to establish the safety of Chr. Hansen's 2'-FL under its intended conditions of use are "generally available" (*i.e.*, in the public domain). From the data and information presented herein, Chr. Hansen concludes their 2'-FL produced with a genetically engineered strain of *E. coli* BL21(DE3) is GRAS for its intended uses in exempt infant formula for preterm infants (specifically preterm post-discharge formula), at levels up to 2.0 g/L as consumed, based on scientific procedures.

7. List of Supporting Data and Information

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Appendix A

FDA's Response Letter to the Supplement for GRN No. 571

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

Re: GRAS Notice No. GRN 000571

Dear Dr. Thompson:

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

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

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

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

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

Potential Labeling Issues

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

Intended Use in Infant Formula

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

Section 301(ll) of FD&C Act

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

Conclusions

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

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

Sincerely,

Susan J. Carlson -S

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

Susan Carlson, Ph.D.
Director
Division of Food Ingredients
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