CHR. HANSEN
Improving food & health

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Food and Drug Administration
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18/05/22

Re: GRAS Notice for the Intended Uses of 3-Fucosyllactose in Infant Formulas, Conventional Foods, and Oral/Enteral Tube Feeding Formulas

Dear Dr. Carlson,

In accordance with 21 CFR 170 Subpart E, Chr. Hansen A/S is notifying the Food and Drug Administration of their conclusion that 3-fucosyllactose (3-FL) produced by fermentation with a genetically engineered *Escherichia coli* BL21(DE3) strain is Generally Recognized as Safe (GRAS) for its intended conditions of use in non-exempt infant formulas, select conventional foods, and oral/enteral tube feeding formulas.

This 3-FL ingredient has been previously notified as GRAS for use in non-exempt infant formulas under GRN No. 925, which was filed by the FDA with "no questions".

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 3-Fucosyllactose (3-FL) in Infant Formulas, Conventional Foods, and Oral/Enteral Tube Feeding Formulas

Chr. Hansen A/S

May 18, 2022

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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
CHOs	Carbohydrates
cGMP	Current Good Manufacturing Practice
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen
EDI	Estimated daily intake
EFSA	European Food Safety Authority
EU	European Union
FALCPA	Food Allergen Labeling and Consumer Protection Act of 2004
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
IbR	Incorporated by Reference
HACCP	Hazard Analysis and Critical Control Point
HMOs	Human milk oligosaccharides
HPAEC-PAD	High performance anion exchange chromatography coupled with pulsed amperometric detection
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
U.S.	United States
USDA	United States Department of Agriculture

1. Signed Statements and Certification

1.1 Statement of Intent

Chr. Hansen A/S ("Chr. Hansen")[1] has previously notified the United States (U.S.) Food and Drug Administration (FDA) of their conclusion that 3-fucosyllactose (3-FL) produced by fermentation with genetically engineered *E. coli* BL21(DE3) strain JBT-3FL is Generally Recognized as Safe (GRAS) for its intended use as an ingredient in cow milk-based, non-exempt infant formula for term infants at 0.44 g/L of formula, as consumed. This GRAS notice was filed by the FDA with "no questions" under GRN No. 925. Chr. Hansen now intends to increase the use level of 3-FL in non-exempt infant formula (including cow-milk-, soy-milk-, and partially hydrolyzed protein-based formulas) to 0.9 g/L, as consumed. Furthermore, Chr. Hansen's 3-FL ingredient is intended for use in additional food categories, similar to those that have been concluded GRAS for 3-FL in a notice submitted by Danisco USA, Inc., which was also filed with "no questions" by the FDA under GRN No. 951[2].

In accordance with 21 CFR 170 Subpart E, Chr. Hansen is submitting this GRAS notice for their 3-FL ingredient produced with genetically engineered *E. coli* BL21(DE3) strain JBT-3FL, which is intended for use in non-exempt infant formula for term infants at 0.9 g/L of formula (as consumed); formula intended for young children aged 1 to 3 years at 1.2 g/L of formula (as consumed); other drinks and foods for infants and young children under 3 years of age at levels ranging from 0.44 to 4.4 g/kg; cereal and nutrition bars, enhanced and "fortified" waters, sports/isotonic and energy drinks, breakfast cereals, fermented milk, flavored milk and mixes, smoothies, yogurts, meal replacement beverages (milk-based and non-milk based), dairy product analogs, fruit juices and nectars (including fruit-based beverages), vegetable juices, and gummy candies at levels ranging from 0.26 to 8.8 g/kg; and in enteral tube feeding formulas at up to 6.6 g/L (as consumed).

Of note, the 3-FL ingredient that is the subject of this GRAS notice is identical to the 3-FL ingredient described previously in GRN No. 925. Chr. Hansen's 3-FL is produced using the same manufacturing processes, including the same *E. coli* BL21(DE3) JBT-3FL production strain, since the completion of GRN No. 925. As such, information related to the technical aspects of the 3-FL ingredient (*i.e.*, identity, method of manufacture, and specifications) can be incorporated by reference from GRN No. 925. To address the changes in the intended conditions of use, a new exposure assessment, together with an updated narrative of the data to support the safety of Chr. Hansen's 3-FL ingredient under the expanded conditions of use, have been compiled for this GRAS notice.

[1] This 3-FL ingredient was initially developed by Jennewein Biotechnology GmbH, which was acquired by Chr. Hansen A/S in 2020.

[2] A GRAS notice has been recently submitted by Glycom A/S under GRN No. 1037 for the intended uses of 3-FL in non-exempt infant formula for term infants at up to 0.75 g/L as consumed; formula and drinks intended for young children (>12 months of age) at up to 2.0 g/L as consumed; foods intended for infants and young children at up to 6.25 g/kg; and various other foods at levels ranging from 1.25 to 25 g/kg. This GRAS notice has not yet been posted in the FDA's GRAS notice inventory and the FDA's response is still pending at the time of preparation of Chr. Hansen's 3-FL GRAS notice.

1.2 Name and Address of Organization

Chr. Hansen A/S
Boege Allé 10-12
2970 Hoersholm
Denmark

1.3 Name of Notified Substance

3-Fucosyllactose (3-FL)

1.4 Intended Conditions of Use

The intended conditions of use for Chr. Hansen's 3-FL are indicated in Table 1.4-1. As mentioned, Chr. Hansen now intends to increase the use level of 3-FL in infant formula to 0.9 g/L (as consumed). Moreover, aside from infant formula, Chr. Hansen's 3-FL is intended for use in additional food categories that are similar to those that have been concluded GRAS under GRN No. 951. The food categories and/or use levels that differ from those in GRN No. 951 are marked in **bold** in Table 1.4-1.

Table 1.4-1 Intended Food Uses and Use Levels for 3-FL in the United States

Food Category (21 CFR §170.3)	**Food Uses[a,b]**	**Use Levels (g/L or g/kg)**
Infant and Toddler Foods	Non-exempt term infant formula (0-12 months)	**0.9[c] (as consumed)**
	Toddler formula (1-3 years)	**1.2[c] (as consumed)**
	Other drinks for young children, incl. yogurt and juice beverages identified as "baby drinks"	0.44
	Hot cereals (dry and RTE)	4.4
	Baby crackers, pretzels, cookies, and snack items	4.4
Beverages and Beverage Bases	Enhanced and fortified waters (incl. flavored and carbonated waters)	0.26
	Non-milk-based meal replacement drinks	**4.0[d]**
	Sports, isotonic, and energy drinks	**0.3[e]**
Breakfast Cereals	Hot Breakfast Cereals (*e.g.*, oatmeal, grits), instant and RTE	6.8
	RTE breakfast cereals	8.8
Dairy Product Analogs	Milk substitutes such as soy milk and imitation milks	0.26
	Non-dairy yogurt	2.64
Grain Products and Pastas	Cereal and granola bars incl. energy, protein, and meal replacement bars	6.6
Milk Products	Fermented milk	0.26
	Flavored milk (incl. RTD and powder mix)	0.26
	Milk-based meal replacement beverages	**4.0[d]**
	Smoothies (dairy and non-dairy)	1.1
	Yogurt	2.64
Processed Fruits and Fruit Juices	Fruit flavored drinks and ades	0.26
	Fruit juices and nectars	0.26

Food Category (21 CFR §170.3)	Food Uses[a,b]	Use Levels (g/L or g/kg)
Processed Vegetables and Vegetable Juices	Vegetable juices	0.26
Soft Candy	**Gummy candies**	**8.8**
Foods for Special Dietary Uses	Oral and enteral tube feeding formulas[f]	**6.6[g] (final product, as consumed)**

Abbreviation(s): 3-FL = 3-fucosyllactose; CFR = Code of Federal Regulations; GRAS = Generally Recognized as Safe; incl. = including; RTD = ready-to-drink; RTE = ready-to-eat.
[a] 3-FL is intended for use in unstandardized products where standards of identity, as established under 21 CFR §130 to 169, do not permit its addition in standardized products.
[b] Additional food categories and/or use levels that have not been previously concluded as GRAS and notified to the U.S. FDA are **bolded**.
[c] 3-FL was previously concluded to be GRAS for use in infant formula (GRN 925, 951) and toddler formula (GRN 951) at a use level of 0.44 g/L.
[d] 3-FL was previously concluded to be GRAS for use in meal replacement beverage at a use level of 1.1 g/L (GRN 951).
[e] 3-FL was previously concluded to be GRAS for use in sports, isotonic, and energy drinks at a level of 0.26 g/L (GRN 951).
[f] Foods for special dietary use were assessed separately from the intended food uses of 3-FL in conventional foods, as they are intended for supplying a particular dietary need. Intake of 3-FL from foods for special dietary use is, therefore, not expected to be cumulative to other dietary sources.
[g] 3-FL was previously concluded to be GRAS for use in foods for special dietary uses at a use level of 4.4 g/L (GRN 951).

1.5 Statutory Basis for GRAS Conclusion

Pursuant to the GRAS final rule [81 Fed. Reg. 54959 (effective 17 October 2016)], Chr. Hansen has concluded that the intended uses of 3-FL, 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 3-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 3-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.

Chr. Hansen A/S
Boege Allé 10-12
2970 Hoersholm
Denmark

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 uses of 3-FL.

1.10 FSIS Statement

Not applicable. 3-FL is not intended for use in product or 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

May 5, 2022

Manki Ho, Ph.D.
Principal Regulatory Affairs Specialist
Chr. Hansen A/S
camaho@chr-hansen.com

Date

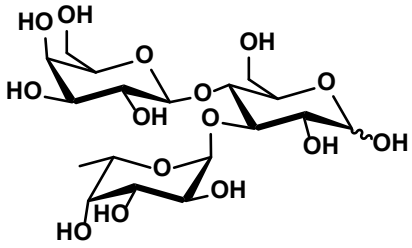
May 5, 2022

Katharine Urbain
Head of Regulatory Affairs – North America
Chr. Hansen A/S
uskaur@chr-hansen.com

Date

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

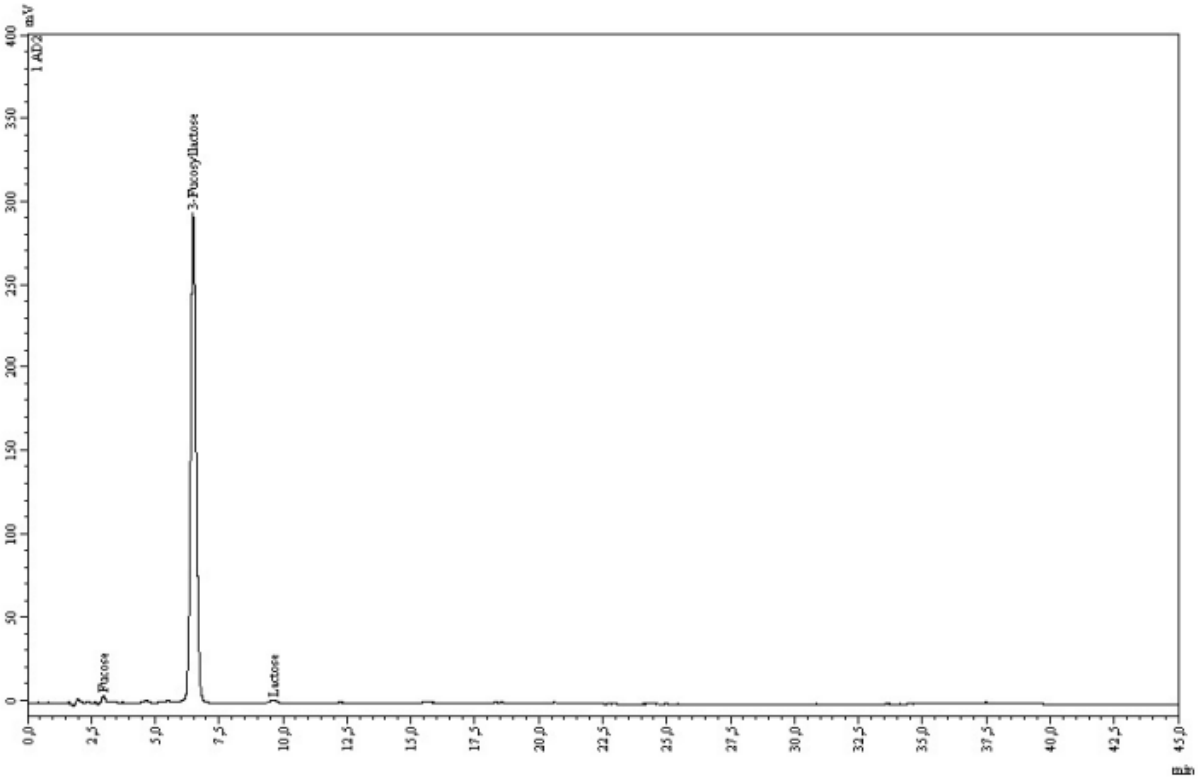
2.1 Identity

Common or Usual Name:	3-Fucosyllactose (3-FL)
Chemical Name:	6-deoxy-α-L-galactohexopyranosyl-(1→3)-[β-D-galactohexopyranosyl-(1→4)]-D-glucohexopyranose
CAS Number:	41312-47-4
Molecular Weight:	488.439 g/mol
Molecular Formula:	$C_{18}H_{32}O_{15}$
Structural Formula:	

3-FL is a fucosylated, neutral trisaccharide consisting of L-fucose, D-galactose, and D-glucose. Chr. Hansen manufactures 3-FL through fermentation with genetically engineered *E. c*oli BL21(DE3) strain JBT-3FL as a processing aid. Details of the analyses conducted to characterize and confirm the identity of the 3-FL ingredient have been described previously in GRN No. 925.

In brief, the structure of Chr. Hansen's 3-FL produced by fermentation has been confirmed using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), ^{1}H nuclear magnetic resonance spectroscopy (NMR), ^{13}C NMR, double-quantum filtered $^{1}H^{1}H$ correlation spectroscopy (COSY), phase-sensitive ^{1}H13C heteronuclear single quantum correlation spectroscopy (HSQC), and phase-sensitive $^{1}H^{13}C$ heteronuclear multiple bond correlation spectroscopy (HMBC). The purified spray-dried powder contains ≥90% 3-FL. Chr. Hansen has established specification limits for the small amounts of residual carbohydrates that may be present in the 3-FL ingredient: lactose (≤5%), glucose (≤3%), galactose (≤3%), and fucose (≤3%). A representative chromatogram of the high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis for Chr. Hansen's 3-FL, as taken from GRN No. 925 (pg. 105), is provided in Figure 2.1-1.

Figure 2.1-1 Representative HPAEC-PAD Chromatogram of Chr. Hansen's 3-FL Ingredient



2.2 Method of Manufacture

The manufacturing process for Chr. Hansen's 3-FL has not changed since GRN No. 925. Accordingly, information related to the production of 3-FL that was presented in GRN No. 925 are incorporated by reference herein and are briefly described below.

2.2.1 Production Strain

Chr. Hansen's 3-FL is produced with genetically engineered *E. coli* BL21(DE3) strain JBT-3FL as a processing aid. The taxonomic classification of the *E. coli* BL21(DE3) parental organism is presented in Table 2.2.1-1.

Table 2.2.1-1 Taxonomic Classification of the Parental Organism

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

As reviewed in previous GRAS notices (e.g., GRN Nos. 485, 571, 925), *E. coli* BL21(DE3) is a safe, non-pathogenic and non-toxigenic bacterium that is often used in various biotechnology applications. *E. coli* is one of the pioneer species to colonize the human gut after birth, being first described in 1885 by Theodor Escherich during the analysis of microbes present in the stool of breastfed infants (reviewed in Shulman et al., 2007). *E. coli* BL21(DE3) is a derivative of the *E. coli* B strain, which is a commensal of the human gut that was isolated from human feces by Félix d'Herelle at the Institut Pasteur in 1918 (Daegelen et al., 2009). Although *E. coli* is a ubiquitous resident of the gastrointestinal tract and is a species of almost exclusively non-pathogenic bacteria, certain pathogenic variants can cause significant diarrheal diseases and other illnesses (Croxen et al., 2013; Martinson & Walk, 2020). Nonetheless, the genome sequence of *E. coli* BL21(DE3) revealed the absence of genes encoding invasion factors, adhesion molecules, and enterotoxins associated with virulence (Jeong et al., 2009). It has also been demonstrated that *E. coli* BL21(DE3) does not carry the well-recognized pathogenic components exhibited by strains of *E. coli* that cause the majority of enteric infections, and it is unlikely to survive in host tissues or to cause disease (Chart et al., 2000). Moreover, in an acute oral toxicity study, gavage administration of endotoxin isolated from *E. coli* BL21(DE3) to male and female CD-1 mice at up to 1,000,000 endotoxin units (EU) per animal did not result in mortalities or signs of toxicity (*i.e*., changes in body weight or abnormal clinical signs) during the 14-day observation period (Harper et al., 2011). As such, *E. coli* BL21(DE3), alongside the closely related *E. coli* K-12 (Studier et al., 2009), are common laboratory strains that are widely used for the overexpression of recombinant proteins. *E. coli* BL21(DE3) is classified as a risk group 1 organism, the lowest possible risk group, according to the German Federal Office of Consumer Protection and Food Safety (BVL).

As described in GRN No. 925, Chr. Hansen introduced a series of genetic modifications into the *E. coli* BL21(DE3) host organism so that it has the ability to synthesize 3-FL using lactose as the starting substrate. A schematic overview of the metabolic pathway for 3-FL synthesis in JBT-3FL is provided in Figure 2.2.1-1, and a listing of the genetic modifications are summarized in Table 2.2.1-2. Notably, JBT-3FL contains a common set of modifications that are also in strains engineered by Chr. Hansen for the manufacture of other human milk oligosaccharides (HMOs), including 2'-fucosyllactose (2'-FL; GRN Nos. 571, 929), lacto-*N*-tetraose (LNT; GRN No. 923), 3'-sialyllactose sodium salt (3'-SL; GRN No. 921), and 6'-sialyllactose sodium salt (6'-SL; GRN No. 922). In addition to the common set of genetic modifications in the "Basic Strain", further deletions and insertions were made to allow for the synthesis of the specific HMO of interest. In the case of JBT-3FL, insertions were introduced to generate the large amounts of GDP-fucose that serve as the fucose donor, and to enable the fucosylation of lactose to produce 3-FL (see Table 2.2.1-2). Additionally, to increase efficiency, genes interfering with the synthesis of 3-FL were deleted or inactivated from the host strain. All genetic modifications are stably integrated into the genome of *E. coli* BL21(DE3). The JBT-3FL production strain does not contain plasmids or other episomal vectors and it is not capable of DNA transfer to other organisms.

JBT-3FL is stored at the production site as glycerol stocks in a master cell bank at -80°C. The glycerol stocks are used to produce working cell banks, which are then used for the production of 3-FL. The 3-FL production strain has been deposited at the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen) German Collection of Microorganisms and Cell Cultures under DSM 33491.

Figure 2.2.1-1 Metabolic Pathway for 3-FL Synthesis in *E. coli* BL21(DE3) Strain JBT-3FL

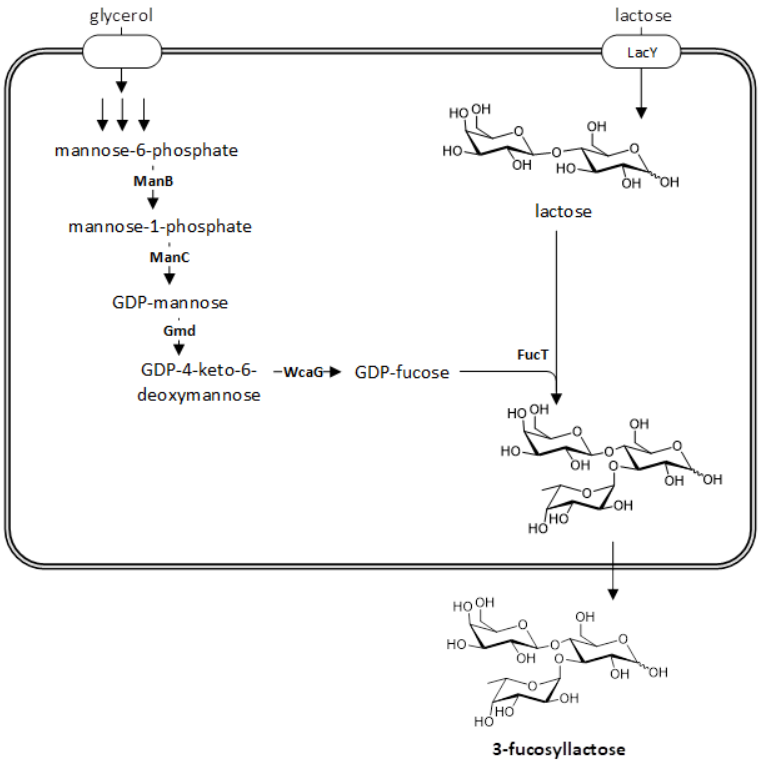


Table 2.2.1-2 List of Genetic Modifications in *E. coli* BL21(DE3) Strain JBT-3FL

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

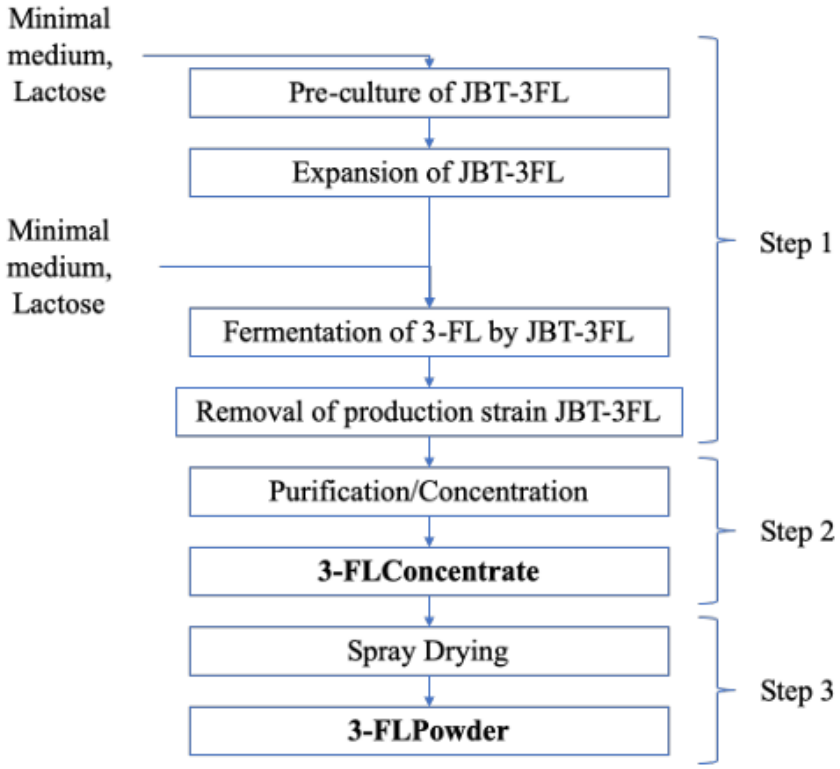
Gene Product Name	Origin of the Gene	Manipulation	Effect
Additional Modifications that are Specific to JBT-3FL			
5'-β-galactosidase	*E. coli* BL21(DE3)	Deletion	No effect (removal of a gene that is non-functional due to a truncation)
Phosphomannomutase	*E. coli* K12	Ectopic expression	To allow for sufficient GDP-fucose production
Mannose-1-phosphate guanosyltransferase	*E. coli* K12	Ectopic expression	
GDP-fucose-4,6-dehydratase	*E. coli* K12	Ectopic expression	
GDP-fucose synthase	*E. coli* K12	Ectopic expression	
α1,3-fucosyltransferase	*Bacteroides fragilis* NCTC 9343	Ectopic expression	To enable the fucosylation of lactose
Antibiotic Resistance Genes			
Dihydrofolate reductase conferring resistance to trimethoprim	*Citrobacter freundii*	Ectopic expression	To allow for the selection of recombinants during genetic engineering.
Bleomycin resistance protein conferring resistance to zeocin	*Streptoalloteichus hindustanus*	Ectopic expression	
[a] The modifications in the "Basic Strain" are also engineered into the production strains used by Chr. Hansen to manufacture other HMO ingredients, including 2'-FL (GRN Nos. 571, 929), LNT (GRN No. 923), 3'-SL (GRN No. 921), and 6'-SL (GRN No. 922).			

2.2.2 Manufacturing Steps

The production of 3-FL is conducted in accordance with current Good Manufacturing Practice (cGMP) consistent with 21 CFR Parts 110 and 117. All of Chr. Hansen's production plants are FSSC 22000-certified and have fully implemented Hazard Analysis Critical Control Point (HACCP) plans, standard operating procedures, and quality control programs to ensure quality of the finished product. As described in GRN No. 925, all raw materials, processing aids, and food contact substances employed in the manufacturing process are suitable for their respective uses in the U.S.

A flowchart of the production process is presented in Figure 2.2.2-1. The fermentation process for 3-FL is conducted in a contained, sterile environment. Batch fermentation is performed in a minimal medium containing a simple, pure carbon source (e.g., glucose, sucrose, glycerol) and the lactose substrate. During fermentation, 3-FL is produced and secreted into the culture medium by the JBT-3FL production strain. Once the fermentation step is completed, the culture medium is separated from the microbial biomass, and 3-FL is further purified from the culture medium through a series of filtration, ion exchange, electrodialysis, and decolorization steps. The resulting 3-FL concentrate is then spray-dried into a powdered product.

Figure 2.2.2-1 Production Process for Chr. Hansen's 3-FL Ingredient



2.2.3 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). Chr. Hansen also 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.

No allergenic materials, as listed in Regulation (EU) No 1169/2011 and FALCPA, are employed in the production of Chr. Hansen's 3-FL other than lactose from cow's milk. Finished food products containing 3-FL marketed in the U.S. will be labeled with appropriate allergen declaration as required under FALCPA.

2.3 Specifications and Analytical Data

To ensure that a consistent food-grade material is produced, Chr. Hansen has established specifications for their 3-FL ingredient. These specifications, which are listed in Table 2.3-1 below, are identical to those presented in GRN No. 925. Data from representative batches of 3-FL demonstrate the manufacturing process reproducibly results in a product that meets the established specifications. Each specification parameter is measured using compendial and/or internally validated, fit-for-purpose methods.

Table 2.3-1 Specifications and Batch Analysis Data for Chr. Hansen's 3-FL Ingredient

Parameter[1]	Method of Analysis	Specification	Batch number		
			16121019	16121029	16121039
Appearance (Color)	Visual[a]	White to ivory-colored	Complies	Complies	Complies
Appearance (Form)	Visual[a]	Spray-dried powder	Complies	Complies	Complies
3-Fucosyllactose	HPAEC-PAD[a,b]	≥ 90 %	96.3	96.9	96.5
Lactose		≤ 5 %	0.7	0.5	0.7
Glucose		≤ 3 %	< LOQ	< LOQ	< LOQ
Galactose		≤ 3 %	< LOQ	< LOQ	< LOQ
Fucose		≤ 3 %	1.1	0.6	< LOQ
Protein	Nanoquant (modified Bradford)[a]	≤ 100 µg/g	< LOQ	< LOQ	< LOQ
Ash	ASU L 06.00-4[c]	≤ 1.0 %	0.36	0.33	0.25
Moisture	KF titration[a]	≤ 9.0 %	6.7	7.4	7.7
Endotoxins	Ph. Eur. 2.6.14[d]	≤ 10 EU/mg	0.007	0.009	0.023
Aflatoxin M1	DIN EN ISO 14501[c]	≤ 0.025 µg/kg	< LOQ	< LOQ	< LOQ
GMO residues	qPCR[e]	Negative	Negative	Negative	Negative
Heavy Metals					
Arsenic	ASU L 00.00-135 – ICP-MS[c]	≤ 0.2 mg/kg	ND	ND	ND
Cadmium		≤ 0.1 mg/kg	ND	ND	ND
Lead		≤ 0.02 mg/kg	ND	ND	ND
Mercury		≤ 0.5 mg/kg	ND	ND	ND
Microbiological Criteria					
Standard Plate Count	ISO 4833-2[c]	≤ 10000 cfu/g	< 10	< 10	<10
Yeast and Mold	ISO 21527-2[c]	≤ 100 cfu/g	< 20	< 20	< 20
Enterobacteriaceae	ISO 21528-2[c]	≤ 10 cfu/g	< 10	< 10	< 10
Salmonella	ISO 6579[c]	Absent/25 g	Absent	Absent	Absent
Cronobacter sakazakii	ISO/TS 22964[c]	Absent/10 g	Absent	Absent	Absent

Abbreviations: 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 quantitation; ND = not detected; qPCR = quantitative polymerase chain reaction; Ph Eur. = European Pharmacopoeia.

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

[b] Carbohydrate by-products with a percent area greater than 0.5% (LOQ) are considered.

[c] Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory. Ash LOQ = 0.01 %; arsenic LOD = 0.05 mg/kg; cadmium LOD = 0.01 mg/kg; lead LOD = 0.01 mg/kg; mercury LOD = 0.005 mg/kg; aflatoxin M1 LOQ = 0.025 µg/kg.

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

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

2.4 Stability

As described in GRN No. 925, to ensure genomic stability and finished product batch-to-batch consistency, all modifications that were introduced into the genetically engineered *E. coli* BL21(DE3) JBT-3FL production strain were stably integrated, and the production of 3-FL occurs in a sterile environment. 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 3-FL. Thus, the production strain is not expected to lose its ability to produce a consistent finished product.

The shelf-life of Chr. Hansen's 3-FL powder is expected to be 2 years from the date of production when stored under ambient conditions.

3. Dietary Exposure

3.1 History of Safe Consumption from Human Milk

3.1.1 HMOs in Human Milk

Human breastmilk is widely recognized as the optimal form of nutrition for infants, with exclusive breastfeeding recommended for the first six months of age, and continued breastfeeding with complementary foods to two years of age and beyond (Eidelman & Schanler, 2012; Kramer & Kakuma, 2012; Pound et al., 2012; WHO, 2021). Human milk contains a highly abundant and unique fraction of structurally diverse glycans (Bode, 2012). These HMOs are present at high concentrations in breastmilk, representing the third most abundant solid component after lactose and total lipids (Bode, 2012, 2019). Total concentrations of HMOs are reported to be highest in colostrum (approximately 20 to 25 g/L) and declining slightly in mature human milk (approximately 15 g/L) as lactation progresses (Soyyılmaz et al., 2021; Thum et al., 2021; Thurl et al., 2017).

All HMOs consist of lactose that is elongated with monosaccharide building blocks, including galactose, N-acetylglucosamine, fucose, and/or N-acetylneuraminic acid (sialic acid), through various glycosidic linkages (Bode, 2019; Walsh et al., 2020a). HMO synthesis occurs in the lactating mammary gland *via* the action of glycosyltransferases, including fucosyltransferases, sialyltransferases, galactotransferases, and acetylglucosaminyltransferases (Bode, 2019; Walsh et al., 2020a). HMOs can be broadly classified into three structural classes: neutral fucosylated HMOs, neutral non-fucosylated HMOs, and acidic HMOs containing sialic acid (Corona et al., 2021; Walsh et al., 2020a). Although over 200 different HMO structures have been identified, a subset of just 15 to 20 HMOs account for the majority of the total oligosaccharide fraction of human milk, with the principal oligosaccharides being fucosyllactoses (including 3-FL), sialyllactoses, LNT, lacto-*N*-neotetraose (LNnT), lacto-*N*-fucopentaoses (I–V) and lacto-*N*-difucohexaoses (I–III) (EFSA NDA Panel, 2014; Soyyılmaz et al., 2021).

Inter-individual variations exist in the types and levels of HMO structures that are present in human milk (Han et al., 2021; Walsh et al., 2020a). Although non-genetic factors (such as lactation stage) may contribute to this variability, maternal genetics (*i.e.*, allelic variations in the Secretor and Lewis genes) is considered a key determinant of the HMO composition of human milk (Bode, 2019; Han et al., 2021; Walsh et al., 2020a). The Secretor gene (*Se*) encodes for an α1-2 fucosyltransferase (FUT2) enzyme, while

the Lewis gene (*Le*) encodes for an α1-3/4-fucosyltransferase (FUT3) (Blank et al., 2012). Genetic variations in the *Se* and *Le* genes affect the expression and functionalities of the FUT2 and FUT3 enzymes, which in turn impact the pattern of fucosylated HMOs observed in human milk (Bode, 2019; Soyyılmaz et al., 2021; Walsh et al., 2020a). Based on the *Se* and *Le* genotypes and corresponding activities of the FUT2 and FUT3 enzymes, four different milk phenotype groups have been described, as summarized in Table 3.1.1-1. Human milk from mothers with inactive FUT2 (*i.e.*, "non-secretors") contain zero to trace amounts of α1-2-fucosylated HMOs such as 2'-FL, while the milk of mothers with inactive FUT3 ("Lewis negative") contain zero to trace amounts of α1-4-fucosylated HMOs (Soyyılmaz et al., 2021). Since α1-3-fucosylation can be catalyzed by other fucosyltransferases (*e.g.,* FUT5, FUT6), production of α1-3-fucosylated HMOs such as 3-FL is not eliminated if FUT3 is inactive, and the presence of 3-FL in human milk is independent of the Secretor and Lewis histo-blood group system (Castanys-Muñoz et al., 2013; Soyyılmaz et al., 2021; Walsh et al., 2020a).

Table 3.1.1-1 Milk Groups According to Lewis and Secretor Status[a]

Milk Group	Classification	*Se* (FUT2)	*Le* (FUT3)	Fucose Linkages	Typical Frequency in the Global Population
1	Lewis Positive, Secretors	+	+	α1-2, α1-3, α1-4	70%
2	Lewis Positive, Non-Secretors	-	+	α1-3, α1-4	20%
3	Lewis Negative, Secretors	+	-	α1-2, α1-3	9%
4	Lewis Negative, Non-Secretors	-	-	α1-3	1%
[a] This Table is adapted from Soyyılmaz et al. (2021) and Walsh et al. (2020).					

3.1.2 Concentrations of 3-FL in Human Milk

While HMOs represent a large component of human milk, they occur only at low concentrations in cow milk, which is commonly used to formulate infant formula (Albrecht et al., 2014). Accordingly, manufactured versions of purified HMOs, including 3-FL, have been developed as ingredients in infant formula as part of ongoing efforts to bring the composition of formula products closer to that of human milk. The intended use levels of Chr. Hansen's 3-FL ingredient in formula products are selected on the basis that they are comparable to naturally occurring concentrations of 3-FL in human milk.

To understand the levels of HMOs that are present in human milk, detailed analyses have been evaluated in a systematic review conducted by Thurl et al. (2017). The concentrations of 3-FL in human milk that were reported from these analyses (mean: 0.44 g/L; 95% confidence limit [CL]: 0.31 – 0.58 g/L) served as the basis for the use level of 3-FL previously concluded as GRAS for inclusion into formula in GRN No. 925 and GRN No. 951 (*i.e.*, **0.44 g/L**). However, although Thurl et al. (2017) was intended as a comprehensive review, there were some important limitations in the methodologies applied by the review authors. For instance, the review excluded publications that: reported only median concentration levels of HMOs, or analyzed milk samples at "*lactation periods not fitting the lactation periods defined in this review*". Additionally, Thurl et al. (2017) calculated the mean concentration of 3-FL based on data reported in milk samples from only secretor mothers. As explained above in Section 3.1.1, the production of α1-3-fucosylated HMOs such as 3-FL is not dependent on Secretor status, and studies have reported the levels of 3-FL to be higher in the milk of non-secretor than secretor mothers (Austin et al., 2016; Cheema et al.,

2022; Thum et al., 2021; Thurl et al., 2010; Wu et al., 2020). Thus, the mean value for 3-FL in human milk (0.44 g/L) calculated by Thurl et al. (2017) may not accurately represent the full range of natural variability in 3-FL levels among the general population, when considering both secretor and non-secretor mothers.

It is also worth noting that during their safety evaluation of 3-FL as a novel food in the European Union (EU), the European Food Safety Authority (EFSA) Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel) recognized the data compiled by Thurl et al. (2017), but adopted different values that they considered to be more appropriate representations of the mean (1.24 g/L) and high (1.44 g/L) concentrations of 3-FL in human milk (EFSA NDA Panel, 2021). These values were taken from Table S2-A of the Thurl et al. (2017) publication, which separated the 3-FL concentration data according to lactation periods (*i.e.*, means and 95% CL were separately reported for lactation days 0–4, 5–10, 11–30, 31–60, 61–100, >100). The highest 3-FL concentrations were reported in milk from lactation day >100, with a mean value of 1.24 g/L and 95% CL of 1.04–1.44 g/L (Thurl et al., 2017). Recognizing the wide variability of 3-FL concentrations in human milk, and that levels of 3-FL actually increase as lactation progresses, the EFSA NDA Panel used the mean concentration of **1.24 g/L** and "high" concentration of **1.44 g/L** to estimate the intake of 3-FL by infants fed human milk. Following the safety assessment by the EFSA NDA Panel, the 3-FL ingredient was authorized in the EU for addition to a range of foods, including infant formula, follow-on formula, and "milk-based drinks and similar products intended for young children" at a maximum level of **0.85 g/L**[3].

More recently, another comprehensive review derived representative HMO concentrations that are reflective of those in pooled milk samples from healthy mothers, in order to provide a more global estimate of HMO levels in human milk throughout lactation, regardless of individual variations resulting from genetic and non-genetic factors (Soyyılmaz et al., 2021). In this review, 57 peer-reviewed primary publications published between 1996 and 2020 that reported the concentrations of individual HMOs in human milk were included for assessment. Since the main focus of the review was to capture the representative mean levels of HMOs within pooled milk samples, for the primary publications where HMO concentrations were reported separately by milk groups or secretor status, conversion factors were applied by the review authors to derive the levels that would be expected in a pooled sample (e.g., assuming 80/20% frequency of secretors/non-secretors in the population). Descriptive statistics were used to summarize the different mean HMO concentrations obtained in each of the studies evaluated in the review (e.g., "mean of means", "maximum mean"). Consistent with other publications (Thum et al., 2021; Thurl et al., 2017; Zhou et al., 2021), the analyses by Soyyılmaz et al. (2021) indicate concentrations of 3-FL in human milk steadily increases from colostrum through late milk ($p<0.0001$). The concentrations of 3-FL were also higher than those reported previously by Thurl et al. (2017), with the mean of means ranging from **0.72 to 0.92 g/L** and maximum mean ranging from **1.67 to 2.57 g/L** across the different lactation periods.

Using a different approach, another recent review article derived weighted means, standard deviations, medians, interquartile ranges, 90th percentiles, and probability distribution using random sampling of the

[3] *Commission Implementing Regulation (EU) 2021/2029 of 19 November 2021 authorising the placing on the market of 3-Fucosyllactose (3-FL) as a novel food under Regulation (EU) 2015/2283 of the European Parliament and of the Council and amending Commission Implementing Regulation (EU) 2017/2470.* DuPont Nutrition & Biosciences ApS is the applicant of this novel food submission, which pertains to 3-FL produced with genetically engineered *E. coli* strain K12 MG1655. This appears to be the same ingredient as the one described in GRN No. 951.

statistical data reported for HMO concentrations in individual primary research papers (Conze et al., 2022). For this review, the analyses were conducted using published concentration data for five HMOs of interest: 3-FL (n=50 publications); 2'-FL (n=36 publications); LNT (n=48 publications); 3'-SL (n=48 publications); and 6'-SL (n=41 publications). Except for 2'-FL, where only data from Secretors were considered, the analyses included all reported statistical data regardless of Secretor status and without separating by lactation phases. For 3-FL, the weighted mean and 90th percentile values were derived at **0.57 g/L** and **1.4 g/L**, respectively, which are comparable to the concentrations reported by Soyyılmaz et al. (2021).

3.1.3 Estimated Intake of 3-FL from Human Milk

An estimation of the daily intake of 3-FL by infants who are exclusively fed human milk is presented in Table 3.1.3-1. These estimates were derived using the concentrations of 3-FL that have been reported in human milk (Conze et al., 2022), and the approximate volume of human milk that is consumed daily by infants (800 to 1,200 mL/day) (EFSA NDA Panel, 2013; Institute of Medicine, 1991).

As discussed in Section 3.1.2, the intended use level of Chr. Hansen's 3-FL in infant formula (0.9 g/L) is within the concentration ranges of 3-FL that have been reported in human milk. Therefore, the estimated intakes of 3-FL from its intended uses will produce similar levels of intakes as those ingested by breastfed infants who consume this same HMO through human milk (see Section 3.2), which helps to support its safety.

Table 3.1.3-1 Estimated Daily Intake of 3-FL in Infants Exclusively Fed Human Milk

Volume of Milk Intake[a]	Concentrations of 3-FL in Human Milk Reported by Conze et al. (2022)		Estimated Daily Intakes of 3-FL from Human Milk[b]
800 mL/day	Mean	0.57 g/L	68 mg/kg bw/day
	90th Percentile	1.4 g/L	167 mg/kg bw/day
1,200 mL/day	Mean	0.57 g/L	102 mg/kg bw/day
	90th Percentile	1.4 g/L	251 mg/kg bw/day

Abbreviation(s): bw = body weight.

[a] The average volume of human milk consumed by infants has been estimated at 800 mL/day, with an upper bound of 1,200 mL/day (EFSA NDA Panel, 2013; Institute of Medicine, 1991).

[b] Calculated as: (Concentration of HMO in Human Milk) * (Milk Volume Ingested) / (Default Body Weight). Default body weight for infants was assumed to be 6.7 kg.

3.2 Estimated Daily Intake of 3-FL from its Intended Uses in the U.S.

3.2.1 Methodology

Estimates for the intake of 3-FL were derived based on the intended food uses and use levels for 3-FL (as described in Table 1.4-1), in conjunction with food consumption data included in the U.S. National Center for Health Statistics' National Health and Nutrition Examination Surveys (NHANES) 2017-2018. The NHANES are conducted as continuous, annual surveys, and they are released in 2-year cycles. During each year of the ongoing NHANES program, individuals from the U.S. are sampled from up to 30 different study

locations in a complex multi-stage probability design intended to ensure the data are a nationally representative sample of the U.S. population.

NHANES 2017-2018 dietary survey data were collected from individuals and households *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2) throughout all four seasons of the year. Day 1 data were collected in-person, and Day 2 data were collected by telephone in the following 3 to 10 days, on different days of the week, to achieve the desired degree of statistical independence. In addition to collecting information on the types and quantities of foods being consumed, NHANES 2017-2018 collected socio-economic, physiological, and demographic information from individual participants in the survey, such as sex, age, body weight, and other variables (such as height and race-ethnicity) that may be useful in characterizing consumption. The primary sample design for NHANES 2017-2018 includes an oversample of non-Hispanic Asian persons, Hispanic persons, non-Hispanic black persons, non-Hispanic white and "other" older persons (≥80 years), and non-Hispanic low income white and "others" persons (≤185% of the Department of Health and Human Services poverty guidelines); however, sample weights were incorporated to allow estimates from these subgroups to be combined to obtain national estimates that reflect the relative proportions of these groups in the population as a whole (CDC, 2022a, 2022b; USDA, 2021b, 2021a).

For the intake assessment, consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of 3-FL by the U.S. population. Statistical analysis and data management were conducted in DaDiet Software (Dazult Ltd.)[4]. Food codes representative of each intended food use were chosen from the NHANES 2017-2018 and were grouped in food use categories according to 21 CFR §170.3. If necessary, product-specific adjustment factors were developed for composite foods/mixtures based on data provided in the Food and Nutrient Database for Dietary Studies (USDA ARS, 2021b, 2021a). Estimates for the daily intake of 3-FL represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2017-2018 (*i.e.*, a value was established for each person). From these average amounts, a distribution was established from which the mean and 90th percentile intake estimates for the cohort of interest were determined, which incorporated survey weights in order to provide representative intakes for the entire U.S. population.

3.2.2 Estimated Daily Intake of 3-FL from its Intended Uses

3.2.2.1 Infant Formula and Conventional Foods

The estimated daily intake (EDI) of Chr. Hansen's 3-FL from its intended food uses in infant formula and conventional foods is provided in Table 3.2.2.1-1 on an absolute basis (g/person/day) and in Table 3.2.2.1-2 on a body weight basis (mg/kg bw/day). The EDI for the intended uses of 3-FL in oral and enteral tube feeding formula were derived separately and are discussed further in Section 3.2.2.2.

The "*per capita*" intake in Table 3.2.2.1-1 and Table 3.2.2.1-2 refers to the estimated intake of 3-FL averaged over all individuals surveyed, regardless of whether they consumed food products in which 3-FL is intended for use, and therefore includes individuals with "zero" intakes (*i.e.*, those who reported no

[4] *DaDiet - The Dietary Intake Evaluation Tool [Software].* (Version 17.04). Straffan, Ireland: Dazult Ltd. Available online: http://dadiet.daanalysis.com.

intake of food products containing 3-FL during the 2 survey days). "Consumer-only" intake refers to the estimated intake of 3-FL by only those individuals who reported consuming food products of interest on either Day 1 or Day 2 of the survey. The percentage of consumers ranged from 64.1% (infants 0 to 6 months old) to 97.8% (toddlers 1 to 3 years old). Since the consumer-only estimates are more relevant to risk assessments as they represent exposures in the target population, only the consumer-only intake results are discussed herein.

Chr. Hansen's 3-FL is intended as an alternative to other sources of 3-FL currently on the U.S. market. As indicated in Table 1.4-1, the intended food uses of Chr. Hansen's 3-FL are highly similar to those previously concluded as GRAS in GRN No. 951. One notable exception is the increased use level of Chr. Hansen's 3-FL In infant formula (from 0.44 g/L to 0.9 g/L) and toddler formula (from 0.44 g/L to 0.9 g/L). Other minor differences include increased use level in other categories (*e.g.*, meal replacement beverages, and sports/isotonic/and energy drinks) and inclusion of additional food categories (*e.g.*, gummy candies). Accordingly, similarities were observed in the EDI values derived. Among the total population (ages 2 years and older), the mean and 90th percentile consumer-only intakes of Chr. Hansen's 3-FL were determined to be 0.7 and 1.6 g/person/day (11 and 26 mg/kg bw/day), respectively. For comparison, the mean and 90th percentile consumers-only intake of 3-FL for the total U.S. population in GRN No. 951 was estimated to be 0.484 and 1.1 g/person/day (9 and 22 mg/kg bw/day), respectively.

On a body weight basis, infants (0 to 6 months, 7 to 12 months) and toddlers (1 to 3 years) had the highest mean and 90th percentile consumer-only intakes. As expected, since the intended use level of Chr. Hansen's 3-FL in infant formula (0.9 g/L) is higher than those concluded as GRAS (0.44 g/L), the EDI for infants are higher than those previously estimated. The mean and 90th percentile consumers-only intake of Chr. Hansen's 3-FL for infants aged 0 to 6 months was determined to be 0.8 and 1.2 g/person/day (123 and 199 mg/kg bw/day), respectively. For infants aged 7 to 12 months, the mean and 90th percentile consumer only intake was 0.9 and 1.7 g/person/day (97 and 187 mg/kg bw/day), respectively. For comparison, in GRN No. 951, the mean and 90th percentile consumers-only intake of 3-FL for infants aged 0 to 6 months was estimated at 0.33 and 0.55 g/person/day (53 and 88 mg/kg bw/day), respectively, and for infants aged 7 to 12 months at 0.374 and 0.682 g/person/day (42 and 77 mg/kg bw/day), respectively.

The intended use level of Chr. Hansen's 3-FL in toddler formula (1.2 g/L) is also higher than those previously concluded GRAS in GRN No. 951 (0.44 g/L). The mean and 90th percentile consumers-only intake of Chr. Hansen's 3-FL for toddlers aged 1 to 3 years were determined to be 0.5 and 1.2 g/person/day (40 and 91 mg/kg bw/day), respectively. For comparison, the mean and 90th percentile consumers-only intakes for toddlers aged 13 to 35 months in GRN No. 951 were estimated at 0.396 and 0.968 g/person/day (33 and 84 mg/kg bw/day), respectively.

As explained in Section 3.1.2 above, the intended use levels of Chr. Hansen's 3-FL in infant formula (0.9 g/L) and toddler formula (1.2 g/L) were selected on the basis that they are within the naturally occurring concentration ranges of 3-FL that have been reported in human milk. Accordingly, the EDI of Chr. Hansen's 3-FL from its intended uses in formula are comparable to the intake of 3-FL consumed by infants who are fed human milk (upwards of 251 mg/kg bw/day; see Section 3.1.3). Moreover, the 90th percentile EDIs for 3-FL by older population groups (18 to 35 mg/kg bw/day) are also well within the estimated intake level for 3-FL by infants consuming this HMO through its natural occurrence in human milk.

Table 3.2.2.1-1 Estimated Daily Intake of 3-FL from its Intended Food Uses in the U.S. (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 mo	0.5	1.1	64.1	120	0.8	1.2
Infants	7 to <12 mo	0.8	1.7	97.0	121	0.9	1.7
Toddlers	1 to 3 y	0.5	1.2	97.8	405	0.5	1.2
Children	4 to 11 y	0.5	1.0	97.6	862	0.5	1.0
Female Teenagers	12 to 19 y	0.4	1.0	82.8	379	0.5	1.1
Male Teenagers	12 to 19 y	0.6	1.7	86.8	371	0.7	1.7
Female Adults	20 y and older	0.5	1.2	77.2	1,654	0.6	1.4
Male Adults	20 y and older	0.7	1.7	75.7	1,482	0.9	2.0
Total Population	2 y and older	0.5	1.5	80.2	5,001	0.7	1.6

Abbreviation(s): mo = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

Table 3.2.2.1-2 Estimated Daily Intake of 3-FL from its Intended Food Uses in the U.S. on a Kilogram Body Weight Basis (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 mo	79	175	64.1	120	123	199
Infants	7 to <12 mo	94	187	97.0	121	97	187
Toddlers	1 to 3 y	39	90	98.1	397	40	91
Children	4 to 11 y	16	35	97.6	860	17	35
Female Teenagers	12 to 19 y	7	16	82.5	372	8	18
Male Teenagers	12 to 19 y	9	21	86.7	368	10	23
Female Adults	20 y and older	7	17	77.1	1,638	9	20
Male Adults	20 y and older	8	21	75.7	1,468	10	24
Total Population	2 y and older	9	23	80.1	4,953	11	26

Abbreviation(s): bw = body weight; mo = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

3.2.2.2 Oral and Enteral Tube Feeding Formula

In GRN No. 951, 3-FL has been previously concluded GRAS for its intended use in oral and enteral tube feeding formula at up to 4.4 g/L (0.88 g/serving). Consistent with GRN No. 951, it is expected that up to three servings of these products may be consumed daily. Thus, the intended uses of Chr. Hansen's 3-FL ingredient in oral and enteral tube feeding formula at 6.6 g/L (1.32 g/serving) corresponds to 3-FL intake of approximately 3.96 g/day. Since these are specialty products that are intended to be consumed to address a specific dietary need, incorporation of 3-FL as an ingredient in oral and enteral tube feeding

formulas are not expected to increase the exposure to 3-FL from its intended uses in other food sources. Additional discussions regarding the intended uses of Chr. Hansen's 3-FL in oral and enteral tube feeding formula are presented in Section 6.4.2 below.

4. Self-Limiting Levels of Use

This Part is not applicable. The intended uses of 3-FL is not self-limiting.

5. Experience Based on Common Use in Food Before 1958

Although there is a history of safe consumption of 3-FL by infants from its presence in human milk, the statutory basis for the conclusion of GRAS status for the intended use of 3-FL is based on scientific procedures, and not common use in food before 1958.

6. Safety Narrative

6.1 Rationale

Chr. Hansen's 3-FL ingredient produced by fermentation with *E. coli* BL21(DE3) strain JBT-3FL has been previously concluded GRAS for its intended use in non-exempt infant formula for term infants at 0.44 g/L, as consumed, which was notified to the U.S. FDA and filed with "no questions" under GRN No. 925. No changes to the manufacturing process or production strain have occurred, and the ingredient meets identical specifications as those described in GRN No. 925.

Chr. Hansen's 3-FL ingredient is highly purified, being specified to contain ≥90% 3-FL. Specification limits have also been established for the small amounts of residual carbohydrates that may be present, namely lactose (≤5%), glucose (≤3%), galactose (≤3%), and fucose (≤3%), which are all components that are widely present in human milk (Newburg, 2013). As described in GRN No. 925, and explained in Section 2.2.1 above, the *E. coli* BL21(DE3) host organism is widely used in biotechnology applications and does not pose any safety concerns. Furthermore, the 3-FL production strain (JBT-3FL) was engineered with genes with known functions, specifically for the purpose of introducing the metabolic pathway for 3-FL biosynthesis. Since the inserted genes do not confer toxicogenicity or virulence, JBT-3FL is also considered as non-toxigenic and non-pathogenic like the *E. coli* BL21(DE3) host organism, which does not carry pathogenic components associated with virulence (Chart et al., 2000; Harper et al., 2011; Jeong et al., 2009). All genetic modifications are stably integrated into the genome of *E. coli* BL21(DE3), and since the JBT-3FL production strain does not contain plasmids or other episomal vectors, it is not capable of DNA transfer to other organisms. Moreover, as described in Section 2.2.2, the production organism is removed through a series of purification steps employed during the manufacturing process of 3-FL, and no recombinant DNA remains in the finished ingredient.

Chr. Hansen's 3-FL has been evaluated in preclinical toxicology studies, including a 90-day oral toxicity study in rats, genotoxicity/mutagenicity studies, and a 21-day tolerance study in neonatal piglets (Hanlon, 2020; Parschat et al., 2020). In these studies, 3-FL was provided as part of a mixture with four other HMO

ingredients (2'-FL, LNT, 3'-SL and 6'-SL), which were all produced by separate microbial fermentation reactions and then subsequently combined. Additionally, the safety of 3-FL is supported by preclinical toxicology studies conducted with a 3-FL test article from other manufacturers that are compositionally and quantitatively similar to Chr. Hansen's 3-FL ingredient (Phipps et al., 2022; Pitt et al., 2019). A summary of these studies is provided in Section 6.3 below.

To identify other published data pertinent to the safety evaluation of Chr. Hansen's 3-FL for its expanded conditions of use, a literature search was conducted through to May 12, 2022. Since the filing of the previous GRAS notices for 3-FL (GRN No. 925 and GRN No. 951), additional comprehensive analyses have been published regarding the concentrations of HMOs (including 3-FL) in human milk (Conze et al., 2022; Soyyılmaz et al., 2021). As described in Section 3.1 above, the analyses suggest the levels of 3-FL in human milk are higher than those previously estimated by Thurl et al. (2017), which were used to establish the intended use level for 3-FL in formula products (0.44 g/L, as consumed) in GRN No. 925 and GRN No. 951. At the intended conditions of use described in this GRAS notice, which includes higher use levels of 3-FL in infant formula (0.9 g/L, as consumed) and toddler formula (1.2 g/L, as consumed), the estimated daily intake for 3-FL in all population groups continues to be within the ranges of 3-FL intakes observed by infants fed human milk (see Section 3.2). Furthermore, since the completion of GRN No. 925, the results of two separate clinical trials in which infants were fed formula containing 3-FL (at 0.75 g/L) have become available (Abbott Nutrition, 2021; Parschat et al., 2021). These studies demonstrate that infant formula containing 3-FL are safe and well-tolerated in infants, as described further in Section 6.4 below.

6.2 Absorption, Distribution, Metabolism, Excretion (ADME)

6.2.1 Overview

The ADME processes of 3-FL have been discussed previously in GRN No. 925 and GRN No. 951, and in the scientific opinion on the safety of 3-FL as a novel food published by the EFSA NDA Panel (EFSA NDA Panel, 2021). As described in these documents, and in other reports by authoritative bodies (EFSA NDA Panel, 2014, 2015, 2019a, 2019b, 2020a, 2020b, 2021; FSANZ, 2021; Health Canada, 2018), it is recognized that HMOs are generally considered non-digestible carbohydrates that undergo limited digestion and absorption in the upper gastrointestinal tract, and instead can be fermented by the intestinal microbiota. A brief summary of the data to support these conclusions is presented in Sections 6.2.2 and 6.2.3 below.

Importantly, considering the structural equivalence of Chr. Hansen's 3-FL to its naturally occurring counterpart in human milk (see Section 2.1), it is expected that the ADME processes of manufactured 3-FL will mimic those in infants consuming the same HMO through human milk.

6.2.2 Preclinical Data

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 intestine. 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).

Data regarding the absorption of 3-FL have also been gathered from toxicology studies conducted in rodents. These studies have been discussed in GRN No. 951, which are incorporated by reference. In an *in vivo* mouse micronucleus assay conducted with 3-FL, plasma concentrations of 3-FL reached 572 and 681 ng/mL in pooled samples from males and females (4 animals/sex), respectively, at 4 hours following the administration of a single gavage dose of 3-FL at 500 mg/kg bw (Pitt et al., 2019). In contrast, 3-FL was not detected in the plasma of the control animals receiving the vehicle (deionized water). Thus, these results suggest some degree of absorption and systemic availability of 3-FL, and demonstrate exposure by the target cells in the rodent bone marrow. In a 90-day sub-chronic oral toxicity study where Crl:CD®(SD) rats were fed diets containing 3-FL at 5% or 10%, blood and urine samples were collected on Day 80 or 81 for pharmacokinetic analyses (Pitt et al., 2019). For the urine collection, animals were placed individually into metabolic cages for 16 h with access to their experimental diets and water. Following urine collection, blood samples were collected at 6 am, 10 am, and 2 pm, from 3 to 4 animals/sex/group at each time point. The intake of 3-FL during this test period was estimated at approximately 2,340 and 4,650 mg/kg bw/day in males and 3,230 and 5,880 mg/kg bw/day in females, for the 5% and 10% test groups, respectively. The serum concentrations of 3-FL (mean ± SD) in male rats ranged from 984 ± 543 to 1520 ± 1200 ng/mL in the 5% test group, and 2080 ± 592 to 2950 ± 669 ng/mL in the 10% test group, while the concentrations in the female rats were approximately 2-fold higher. However, although measurable levels of 3-FL were detected in serum, the extent of systemic exposure is considered to be low. The amount of 3-FL recovered in the urine, as a mol percentage of the daily dietary intake, was reported at 0.39% (males) and 0.41% (females) for the 5% test group, and at 0.35% (males) and 0.36% (females) for the 10% test group. Accordingly, the study authors concluded that: "*Quantitation of 3-FL in serum and urine confirms negligible systemic exposure with absorption well below 1.0% of daily dietary intake*."

6.2.3 Human Data

Data in the literature suggest a small fraction of HMOs can be absorbed intact and excreted in the urine. HMOs have been detected in the plasma and urine of breastfed infants (Chaturvedi et al., 2001; Dotz et al., 2014, 2015; Goehring et al., 2014; Marriage et al., 2015; Obermeier et al., 1999; Rudloff et al., 1996, 2012; Ruhaak et al., 2014). However, the concentrations detected were low when compared with those in human milk. In one study involving 16 infant-mother dyads, measurable amounts of 3-FL were detected in the plasma and urine samples of the breastfed infants (Goehring et al., 2014). The absolute amount of 3-FL in the samples was not quantified in this study; however, based on the absolute quantification of 2'-FL (an isomer of 3-FL) and 6'-SL, the study authors reported the relative fraction of absorbed HMOs to be low, with concentrations in plasma and urine accounting for 0.1% and 4%, respectively, of the amount ingested from breastmilk (Goehring et al., 2014). Other studies have similarly estimated that approximately 0.5 to 1.5% of the ingested HMOs are absorbed and excreted in the urine (Chaturvedi et al., 2001; Marriage et al., 2015; Obermeier et al., 1999; Rudloff et al., 1996, 2012).

In breastfed infants, it has been reported that approximately 40 to 50% (Coppa et al., 2001), and as high as 97% (Chaturvedi et al., 2001), of the ingested HMOs are excreted in the feces. Using a breath hydrogen test in infants, it was demonstrated that HMOs (consumed as a purified oligosaccharide fraction from human milk) can undergo fermentation in the colon (Brand-Miller et al., 1998). The dominant gut bacteria

in infants, *e.g. Bifidobacterium* and *Bacteriodes* species, have specific enzymes (glycosidases) and transporters that allow for the cleavage and utilization of HMOs as growth factors, thereby generating metabolites such as short-chain fatty acids (acetic, propionic, and butyric acids) and lactate in the process (EFSA NDA Panel, 2014; Masi & Stewart, 2022; Walsh et al., 2020b).

6.3 Toxicology Studies

6.3.1 Studies Conducted with Chr. Hansen's 3-FL

6.3.1.1 Genotoxicity/Mutagenicity – *IbR from GRN No. 925*

Chr. Hansen's 3-FL, as part of a mixture of five HMOs, has been evaluated in a bacterial reverse mutation assay and an *in vitro* micronucleus assay. The results of these studies have been published by Parschat et al. (2020) and they are described in detail in GRN No. 925, which is incorporated by reference. The test article employed, termed "5HMO-Mix", comprised 2'-FL (47.1% dry weight), 3-FL (16.0% dry weight), LNT (23.7% dry weight), 3'-SL (4.1% dry weight), 6'-SL (4.0% dry weight), and other minor carbohydrates (5.1% dry weight).

In the bacterial reverse mutation assay, which was compliant with OECD Guideline No. 471 and GLP, two independent experiments were conducted with strains of *Salmonella* Typhimurium (TA98, TA100, TA102, TA1535, and TA1537). Each were conducted in triplicates, with and without metabolic activation. The first experiment was conducted as a plate incorporation test and the second as a preincubation test. The 5HMO-Mix was applied at concentrations of 5.0, 10.0, 31.6, 100, 316 or 600 mg/plate. No cytotoxicity or mutagenicity were noted in any of test strains at the highest concentrations tested in either the plate incorporation or preincubation tests. Therefore, it can be concluded that 5HMO-Mix was not mutagenic under the conditions of the assay.

The *in vitro* micronucleus assay was conducted with 5HMO-Mix in accordance with OECD Guideline No. 487 and GLP (Parschat et al., 2020). Human peripheral blood lymphocytes were incubated in medium containing the 5HMO-Mix at concentrations of 7.5, 15, 30, and 60 mg/mL for 4 or 24 hours, in the presence and absence of metabolic activation. No chromosomal damage was observed with 5HMO-Mix under the conditions tested, and 5HMO-Mix was concluded to be not genotoxic based on the results of this assay.

6.3.1.2 Sub-Chronic Oral Toxicity – *IbR from GRN No. 925*

Chr. Hansen's 3-FL, as part of a mixture of five HMOs, has been evaluated in a 13-week oral toxicity study compliant with OECD Guideline No. 408 and GLP (Parschat et al., 2020). The 5HMO-Mix test article was identical to those used for the genotoxicity/mutagenicity assay, comprising 2'-FL (47.1% dry weight), 3-FL (16.0% dry weight), LNT (23.7% dry weight), 3'-SL (4.1% dry weight), 6'-SL (4.0% dry weight), and other minor carbohydrates (5.1% dry weight).

The results of these studies have been described in detail in GRN No. 925, which is incorporated by reference. In brief, administration of 5HMO-Mix at 10% in the diet of female CD rats was well tolerated in an initial 7-day pilot study, and this dietary concentration was selected for the 13-week oral toxicity study. For this latter study, CD rats (10/sex/group) were fed a control diet, or the same diet containing 10% of the 5HMO-Mix *ad libitum* for 91 days. No test item-related changes were observed for animal

behavior or external appearance, nor were there any relevant changes with respect to detailed clinical observations, ophthalmological examinations, neurological parameters, body weight, body weight gain, body weight at autopsy, food and drinking water consumption, hematological, clinical chemistry, urinalysis, macroscopic inspection at necropsy, the majority relative and absolute organ weights, or the myeloid/erythroid ratio in the bone marrow. Although some statistically significant changes were noted in body temperature, motility, neutrophilic granulocytes, selected clinical chemistry parameters, absolute and relative organ weights (brain and kidneys), and the specific gravity of the urine in the 5HMO-Mix-treated animals, all deviations were limited to one sex, within the historical range for the laboratory, generally below 20%, and deemed to be not related to 5HMO-Mix. Additionally, the histopathological examination also revealed no test item-related morphological changes at the end of the 91-day treatment period. A mild increase in the incidence of hepatocellular lipid was limited to male rats in the test group, which again was not consistent with other study findings, and therefore not considered related to the 5HMO-Mix.

The NOAEL for 5HMO-Mix in this study was concluded to be 10% in the diet, which is equivalent to intakes of 5HMO-Mix at 5.67 g/kg bw/day for males and 6.97 g/kg bw/day for females. Considering the 5HMO-Mix test article contains 16.0% dry weight of 3-FL, this corresponds to intakes of 0.91 g 3-FL/kg bw/day for males and 1.12 g 3-FL/kg bw/day for females.

6.3.1.3 Tolerance Study in Neonatal Piglets – *IbR from GRN No. 925*

Chr. Hansen's 3-FL, as part of a mixture of five HMOs, was administered to 2-day-old Yorkshire crossbred piglets for 21 days. The results of this study have been described in detail in GRN No. 925, and they have since been published by Hanlon (2020). In brief, 36 experimentally naïve domestic two-day-old Yorkshire crossbred piglets (n=12/group) were assigned to receive one of the following treatments: a control diet; a diet containing 5.75 g/L of 5HMO-Mix; or a diet containing 8.0 g/L of 5HMO-Mix. The control diet was Land O'Lakes ProNurse® Specialty Milk Replacer, which was also used as the base for both of the 5HMO-Mix test diets. The 5HMO-Mix used in this study 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.

There were no treatment-related differences in body weight, food consumption, or feed efficiency among 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 5HMO-Mix, nor were there any findings in organ weights, or macroscopic and microscopic inspection of tissues that could be attributed to 5HMO-Mix. 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. Furthermore, the author noted that increased colon and cecum weights have been observed in studies with other oligosaccharides, and these findings are considered to be an adaptive response rather than an adverse effect. 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 this male piglet 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 this piglet. 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 5HMO-Mix, 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 5HMO-Mix to neonatal piglets for 21 days at concentrations up to 8.0 g/L in milk replacer was well-tolerated and did not produce adverse effects on growth and development. For the high-dose group (8.0 g/L), this corresponds to calculated intakes of 3.6 and 3.7 g 5HMO-Mix/kg bw/day in males and females, respectively. Considering the 5HMO-Mix test article contained 10.4% 3-FL by dry weight, this corresponds to 3-FL intakes of approximately 0.37 g 3-FL/kg bw/day in males and females.

6.3.2 Studies Conducted with 3-FL from Other Manufacturers

6.3.2.1 Overview

Toxicology studies have been conducted using 3-FL produced by fermentation with a genetically engineered *E. coli* K12 MG1655 strain, which is manufactured by DuPont Nutrition & Biosciences. These studies, including a range of genotoxicity/mutagenicity assays and a 90-day oral toxicity study, have been published by Pitt et al. (2019). The results of these studies have been described in GRN No. 925 and 951, which are incorporated by reference. Additionally, an unpublished 6-day and 21-day piglet study have since been described in an EFSA scientific opinion titled *Safety of 3-FL (3-Fucosyllactose) as a novel food pursuant to Regulation (EU) 2015/2283* (EFSA NDA Panel, 2021). The test article employed in these studies contained 94.6% 3-FL, as well as residual lactose (1.5%), glucose/galactose (1.3%), fucose (1.2%), and other minor carbohydrates (1.4%) (EFSA NDA Panel, 2021; Pitt et al., 2019).

Additionally, since the filing of GRN No. 925 and 925, toxicology studies have been published for 3-FL produced by fermentation with genetically engineered *E. coli* K-12 DH1 strain, which is manufactured by Glycom A/S. These studies, which include genotoxicity/mutagenicity assays and a 90-day oral toxicity study, have been published by Phipps et al. (2022). The test article employed in these studies comprised 94.63% 3-FL, along with residual lactose (0.36%), 3-fucosyl-lactulose (0.18%), fucose (0.23%), and other carbohydrates (0.99%).

As presented in Section 2.3, analyses from three representative production batches of Chr. Hansen's 3-FL ingredient demonstrate that it contains primarily 3-FL (96.3 to 96.9%), with small amounts of residual lactose (0.5 to 0.7%) and fucose (<0.5 to 1.1%) (see Table 2.3-1). The specifications for Chr. Hansen's 3-FL ingredient include limits for residual glucose (≤3%) and galactose (≤3%), though these monosaccharides were below the limit of quantitation (<0.5%) in the three batches analyzed (see Table 2.3-1). Given the carbohydrate profiles of the 3-FL preparations are similar, the results of these studies can be considered corroborative in supporting safety of Chr. Hansen's 3-FL. These studies are discussed in Sections 6.3.2.2 to 6.3.2.4 below.

6.3.2.2 Genotoxicity/Mutagenicity

3-FL produced with genetically engineered *E. coli* K12 MG1655 has been evaluated in an OECD-compliant bacterial reverse mutation test, *in vitro* mammalian chromosomal aberration test, *in vitro* mammalian cell micronucleus test, and a confirmatory *in vivo* mouse micronucleus assay (Pitt et al., 2019). A summary of these studies is presented in Table 6.3.2.2-1. As discussed in GRN No. 925 and 951, a repeatable

statistically significant trend towards higher incidences of micronuclei was observed at the 4-h activated test conditions, at ≥2,500 µg/mL in the first assay and ≥1,000 µg/mL in a second confirmatory assay. However, the actual incidences of the micronuclei in all treated groups were within the 95% confidence interval (CI) of the laboratory historical control database. The results of this assay were concluded to be "equivocal" by Pitt et al. (2019), who also noted that "*micronucleus assay with CHO cells can produce false positive rate up to 53%, most likely due as a result of p53 deficiency in these cell lines*". No evidence of genotoxicity was observed in a subsequent *in vivo* micronucleus test conducted with 3-FL in mice (Pitt et al., 2019).

Additionally, an OECD-compliant bacterial reverse mutation test and *in vitro* mammalian cell micronucleus test have been conducted with 3-FL produced with a genetically engineered *E. coli* K-12 DH1 strain (Phipps et al., 2022). A summary of these studies is also presented in Table 6.3.2.2-1. The results of both assays were considered negative, and the study authors concluded the 3-FL preparation is not mutagenic and not genotoxic.

Table 6.3.2.2-1 Genotoxicity/Mutagenicity Assays Conducted with 3-FL Produced by Other Manufacturers

Study	Test System	Concentrations of 3-FL	Main Conclusion
Pitt et al., 2019 - *IbR from GRN No. 925 and GRN No. 951*			
Bacterial reverse mutation test	• *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2uvrA • ± metabolic activation (S9)	0, 333, 667, 1000, 3333, 5000 µg/plate	3-FL is not mutagenic under the conditions tested.
in vitro mammalian chromosomal aberration test	• Cultured human lymphocytes • Conducted in the absence of metabolic activation (S9) for the 24h incubation, ± S9 for the 4h incubation	0, 1250, 2500, 5000 µg/mL	3-FL is not clastogenic under the conditions tested.
in vitro mammalian cell micronucleus assay	• Chinese Hamster Ovary (CHO-K1) cells • Conducted in the absence of metabolic activation (S9) for the 24h incubation, ±S9 for the 4h incubation	0, 500, 1000, 2500, 3500, 5000 µg/mL	Study was concluded to be equivocal (neither positive nor negative).
In vivo mouse micronucleus assay	• 8-week old Crl:CD1(ICR) mice (at least 5/sex/group) • Blood samples collected 48 and 72 hours post-dosing for analyses of micronuclei in peripheral blood reticulocytes	Single oral gavage of 0, 500, 1000, 2000 mg/kg bw	No statistically significant or biologically relevant increases in the micronucleated reticulocyte frequency were observed in mice administered 3-FL.
Phipps et al., 2022			
Bacterial reverse mutation test	• *Salmonella* Typhimurium strains TA98, TA100, TA1535, TA1537 and *E. coli* strain WP2uvrA • ± metabolic activation (S9)	Plate incorporation method: 0, 5, 15, 50, 150, 500, 1500, or 5000 µg/plate Pre-incubation method: 0, 50,	3-FL is not mutagenic under the conditions tested.

Study	Test System	Concentrations of 3-FL	Main Conclusion
		150, 500, 1500, or 5000 µg/plate	
in vitro mammalian cell micronucleus assay	• Human lymphocytes • Conducted in the absence of metabolic activation (S9) for the 20h incubation, ±S9 for the 3h incubation	0, 500, 1000, 2000 µg/mL	3-FL is not clastogenic or aneugenic in this test.

6.3.2.3 Acute and Sub-Chronic Oral Toxicity

3-FL Produced with *E. coli* K12 MG1655 Derived Strain – ***IbR from GRN No. 925 and GRN No. 951***

In an acute oral toxicity study (OECD-compliant), five female Crl:CD®(SD) rats were administered a single bolus dose of 3-FL produced with *E. coli* K12 MG1655 at 5,000 mg/kg bw by gavage (Pitt et al., 2019). No deaths or clinical signs of toxicity occurred over the 14-day observation period, and there were no macroscopic observations at necropsy.

3-FL produced with *E. coli* K12 MG1655 has also been evaluated in OECD-compliant sub-chronic oral toxicity study (Pitt et al., 2019). In this study, male and female Crl:CD®(SD) rats (10/sex/group) were fed either a basal diet, or the same diet containing one of the following on a w/w basis: 5% 3-FL, 10% 3-FL, or 10% fructo-oligosaccharides (FOS). The diets were consumed *ad libitum* for at least 90 consecutive days. All of the animals survived through the study period. There were no ophthalmological findings, clinical or physical observations, or effects on neurobehavioral parameters attributable to consumption of diets containing 3-FL. Administration of 3-FL at 5% and 10% dietary concentrations did not produce any statistically significant or biologically relevant differences in final body weight, overall body weight gain, feed consumption, or feed efficiency. A significantly higher mean cell volume (MCV) and mean cell hemoglobin (MCH) were observed in the 5% 3-FL males ($p<0.05$). However, this response was not dose-related and was not associated with changes in any other hematological parameters. There were no statistically significant differences in coagulation or quantitative urinalysis parameters. No statistically significant biologically or toxicologically relevant differences in organ weights, macroscopic, or microscopic findings were observed among groups. As such, the NOAEL from this study was concluded as 10% 3-FL in the diet, which corresponds to average intakes of 5.98 and 7.27 g/kg bw/day in male and female rats, respectively.

3-FL Produced with *E. coli* K-12 DH1 Derived Strain

A repeated dose 14-day oral dose range finding study was first conducted in which neonatal rats (8/sex/group) were administered 3-FL at 0 (vehicle control), 3,000, or 4,000 mg/kg bw/day once daily by gavage for 14 days starting from Day 7 of age (Phipps et al., 2022). The 4,000 mg/kg bw/day dose was considered to be the maximum feasible dose based on the solubility in the vehicle (*i.e.,* sterile water). No mortalities were observed and there were no adverse clinical signs. Although skin reddening and yellow staining around the anus/perianal region were observed for some of the animals administered 3-FL, these observations were not considered adverse as they were transient and largely absent by the end of the

study. Animals in the 3-FL groups had similar body weight gain as the control animals, and no macroscopic findings were observed at necropsy. Thus, the 4000 mg/kg bw/day dose was concluded to be well-tolerated, and it was selected as the high-dose for the 90-day oral toxicity study.

The 90-day oral toxicity study was conducted in accordance with OECD Test Guideline 408, but modified to commence dosing of the animals at 7 days of age (*i.e.*, 2 weeks before weaning). Groups of neonatal rats (10/sex/group) were administered 3-FL by gavage at 0 (water control), 1,000, 2,000, or 4,000 mg/kg bw/day for 90 consecutive days. Another group of animals (10 males, 10 females) received oligofructose at 4,000 mg/kg bw/day as a reference control. The vehicle control group, reference control group, and high-dose group (4,000 mg/kg bw/day) included a set of additional animals (5/sex/group) who were dosed for 90-days and then followed during a 4-week recovery period. No mortalities were observed, and there were no adverse clinical signs or abnormal developmental indices. Similar to the 14-day dose-range finding study, skin reddening and yellow staining around the anus/perianal region were observed for some of the animals in the high-dose 3-FL group and reference control group, but these findings were transient and primarily observed before weaning. No test-item related ocular findings were observed during ophthalmological examination at Week 13. Although minor statistically significant differences in body weight were observed at sporadic timepoints during the study, the study authors noted that the final body weights, body weight gain, and food consumption were similar across all groups.

With respect to hematology and clinical biochemistry parameters, the only statistically significant differences observed were a shortened activated partial thromboplastin time in the low-dose males, and a slight increase in sodium for mid-dose males, when compared to the vehicle control. In the absence of a dose-response relationship, these observations were considered unrelated to the test article by the study authors. Additionally, no test article-related adverse effects were observed for urinalysis parameters. When compared to the vehicle control, some statistically significant changes were observed, including increased urinary pH (high-dose males), decreased specific gravity (low-dose males, both sexes at the mid- and high-dose groups, both sexes in reference control), and decreased urinary protein, creatinine, and glucose (high-dose males, reference control males). However, such changes were also similarly observed in the oligofructose reference control group. Moreover, no statistically significant differences were observed among groups for any urinalysis parameter by the end of the recovery period. Upon necropsy, although some statistically significant differences in organ weights were reported for animals in the 3-FL groups compared to the vehicle control (lower relative weight for the brain and kidneys, and increased relative weight for the liver), the study authors noted that these were considered unrelated to the test article as they were not associated with a clear dose-response. There were also no test-article related macroscopic or histopathological findings, with those observed being typical background findings for animals of this age and strain. Thus, the study authors concluded the NOAEL for 3-FL to be 4,000 mg/kg bw/day, the highest dose tested.

6.3.2.4 Piglet Studies

3-FL produced with *E. coli* K12 MG1655 have been evaluated in 6-day and 21-day studies conducted with preweaning Landrace crossbred swine farm piglets. Although these studies are unpublished, the data have been reviewed by the EFSA NDA Panel and a brief summary of the study results are publicly available (EFSA NDA Panel, 2021).

The test article employed contained a 3-FL content of 95%. A preliminary 6-day study was conducted where two male and two female piglets were given a dose of ~975 mg 3-FL/kg bw/day (500 mL/kg bw per day of 2 g/L 3-FL in milk replacer offered *via* dish feeding). No deaths or adverse effects on clinical observations, body weight, food consumption and food efficiency, clinical pathology or macroscopic observations were noted in any of the animals. In the main 21-day GLP study, six male and female neonatal piglets were given 1 or 2 g/L of 3-FL in 500 mL/kg bw (in milk replacer six times a day), corresponding to an approximate average dose of 450 and 900 mg/kg bw/day at the two dose levels of 3-FL. An additional group of animals received FOS at 2 g/L as a comparator. According to EFSA NDA Panel (2021), the following results were observed: "*No deaths or clinical signs were noted. A slight non-statistically significant reduction in food consumption and body weight was observed for piglets receiving the highest dose of 3-FL, mainly in males. A slight variation in some haematological parameters was recorded at 2 g 3-FL/L in males on Day 22, related to red blood cells (with statistical significance for the mean corpuscular volume* [MCV] *and haemoglobin concentration). Small statistically significant decrease in alkaline phosphatase* [ALP] *was noted with some time and dose correlation, mainly at the dose of 2 g/L for both 3-FL and fructooligosaccharide. In males at the highest 3-FL dose, a small decrease in globulins was also recorded. No other variations in chemical chemistry and coagulation parameters were noted. No gross pathology or histological alterations were noted. Although no clear test article-related findings were recorded and the administered doses appeared well tolerated, there were signs considered related to a reduced food intake at the highest dose of 3-FL in male piglets*."

Based on the results of the study, as well as the toxicology studies published by Pitt et al. (2019), the EFSA NDA Panel concluded that 3-FL is safe for its proposed conditions of use in a variety of foods, which included infant and follow-on formula (at 0.85 g/L, as consumed), other foods for infants and toddlers, and foods for special medical purposes. These uses have been approved in the EU under *Commission Implementing Regulation (EU) 2021/2029 of 19 November 2021 authorising the placing on the market of 3-Fucosyllactose (3-FL) as a novel food under Regulation (EU) 2015/2283 of the European Parliament and of the Council and amending Commission Implementing Regulation (EU) 2017/2470.*

6.4 Clinical Studies

6.4.1 Studies with Chr. Hansen's 3-FL in Infants

NCT03513744

A multi-centered, randomized, double-blinded, controlled, parallel group clinical study was conducted to evaluate the safety and tolerability of a blended mixture of five HMO ingredients manufactured by Chr. Hansen (ClinicalTrials.gov identifier: NCT03513744). The five HMOs, which included 3-FL together with 2'-FL, LNT, 3'-SL and 6'-SL (termed "5HMO-Mix"), were all produced by separate microbial fermentation reactions and then subsequently combined. The results of this clinical study have been published (Parschat et al., 2021).

Healthy term infants ≤14 days of age were randomized to receive an infant formula containing 5HMO-Mix (n=113) or a control infant formula (n=112) for 4 months. A group of exclusively breastfed infants (BM) was also included as a reference control (n=116). The 5HMO-Mix provided 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, which was achieved by partially replacing some of the

carbohydrates (*i.e.*, maltodextrin) in the control formula. The control formula contained the same quantities of proteins, lipids, vitamins and other nutrients as the formula formulated with 5HMO-Mix. The study comprised six visits (V1–V6) throughout the intervention period, followed by an 8-week voluntary period where infants continued to receive their assigned formula. If the parents agreed to continue during the 8-week voluntary follow-up period, V7 was scheduled at 6 months ± 7 days after V1.

The primary outcome was mean daily body weight gain over a 4-month period. In the 5HMO-Mix group, the mean increase in body weight from V1 to V6 was 3347.7 ± 667.0 g (full-analysis set; FAS) and 3329.8 ± 670.9 (per protocol set; PPS) with a daily mean body weight increment of 29.9 ± 5.8 g/day (FAS) and 29.8 ± 6.0 g/day (PPS). The two-sided 95% CI ranged from –0.7 to 2.4 g/day (FAS) and from –0.8 to 2.3 g/day (PPS), with the lower bound above the non-inferiority margin of –3 g/day in both populations. Therefore, formula supplemented with 5HMO-Mix was considered non-inferior to the control formula with respect to mean daily body weight gain. There were no significant differences in mean weight, length or head circumferences among the 5HMO-Mix and control formula groups during the study. There were also no significant differences in the weight-for-age, length-for-age, or head circumference-for-age z-scores among the 5HMO-Mix and control formula groups. When compared to the BM group, the 5HMO-Mix and control formula groups had slightly higher body weight and weight-for-age z-scores at V6, as well as slightly higher body length and length-for-age z-scores at V5 and V6 that reached statistical significance in the FAS. The study authors noted that faster weight gain has previously been reported for infants bottle-fed with formula, a finding that is also observed when human breastmilk is fed by bottle and may be associated with loss of the infant's self-regulation of energy intake due to early bottle feeding. Furthermore, the study authors pointed out that infant formula containing the 5HMO-Mix and the control infant formula used in this study had energy values of ~68 and ~69 kcal/dL, respectively, and therefore a slightly higher mean energy value than human breastmilk.

The calculated two-sided 95% CIs for the total incidence of adverse events (AEs) and serious adverse events (SAEs) were comparable in all three study groups. The total incidence of AEs was similar in the 5HMO-Mix and control formula groups, with both being slightly higher compared to the BM group. The 5HMO-Mix group had 335 AEs reported in 83 (80.6%) infants, while 289 AEs were reported for 84 (80.8%) infants in the control formula group, and 191 AEs were recorded for 73 (70.2%) infants in the BM group. The number and intensity (mild, moderate, severe) of the reported events were similar among the three groups. The total number of reported AEs was 815, among which 50 were deemed related to the study product (relationship is possible, probable, definite or not assessable/missing), and these were equally distributed in the 5HMO-Mix and control formula groups. A slightly higher incidence of atopic dermatitis was reported in the control formula group (n = 7) compared to the 5HMO-Mix group (n = 0; p = 0.0141), whereas more genital fungal infections were reported in the 5HMO-Mix group (n = 5) compared to the control formula group (n = 0; p = 0.0290). Hematochezia (blood stains in stool) was more frequent in the 5HMO-Mix group, occurring in 7 infants (6.8%) in 5HMO-Mix group; 2 infants in control formula group (1.9%), 2 infants in the BM group (1.9%). Although hematochezia was more frequently reported in the 5HMO-Mix group, the study authors noted that the overall frequency was still very low, and there were no specific safety concern indicators. The hematochezia could be explained by anal fissures, transient firm stool consistency, dyspepsia, gastroenteritis due to contact with pathogenic bacteria or viruses, or allergy to bovine milk protein. Therefore, the study authors indicated a relationship between the frequency of hematochezia and HMO intake cannot be assumed.

Sixteen serious AEs (SAEs) were reported during the study. Three SAEs were reported in the 5HMO-Mix group, representing 2.9% of the infants, compared to nine (7.7%) in the control formula group and four (3.8%) in the BM group. Four of the SAEs (in four infants) were deemed related to the investigational product, with two SAEs occurring each in the 5HMO-Mix and control formula groups. One infant receiving the 5HMO-Mix was hospitalized due to choking and gastroesophageal reflux but recovered. The formula was not withdrawn and the infant continued in the study. The second infant in the 5HMO-Mix group experienced severe diarrhea, the formula was withdrawn, and the infant was treated with hydrolyzed milk before leaving the study. Both SAEs in the control group resulted in diagnosis of suspected allergy to bovine milk protein. The infants recovered after treatment with saline and hydrolyzed milk but left the study prematurely.

For the gastrointestinal tolerance parameters, no differences were observed among the three groups in the frequency of flatulence. The mean total score for regurgitation was slightly higher in the 5HMO-Mix group compared to the control formula group at all visits except V2, and compared to the BM group at V1. At all other time points though, the regurgitation scores between the HMO-Mix and BM groups appeared comparable. Vomiting was similar in both formula groups at all time points but was less frequent in the BM group at V1, V2, and V3. Generally, no differences in stool consistency were observed between the two formula groups. Soft stools were observed significantly more frequently in the 5HMO-Mix group compared to the control formula group at V1 to V4; however, the number of soft stools in the BM group exceeded those of both formula groups at nearly all time points. The mean number of formed stools per subject at each visit was similar in all three groups throughout the intervention.

Based on the results of this study, the investigators concluded that an infant formula containing HMOs, including 0.75 g/L of 3-FL, supports normal infant growth and is safe and well tolerated by healthy term infants.

NCT04105686

Abbott Nutrition completed a growth monitoring study (ClinicalTrials.gov identifier: NCT04105686) that compared the growth of infants receiving a milk-based experimental formula that contained a mixture of five HMOs manufactured by Chr. Hansen (including 0.75 g/L of 3-FL) 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 3-FL was safe, well tolerated, and supported normal growth of infants.

6.4.2 Data to Support the Use of 3-FL in Oral and Enteral Tube Feeding Formulas

As described in GRN No. 951, 3-FL has been concluded GRAS for its intended use in oral and enteral tube feeding formula at up to **4.4 g/L** (**0.88 g/serving**). Of note, 2'-FL (which is a structurally-related isomer of 3-FL) has also been concluded GRAS for use in oral and enteral tube feeding formula at up to (**20 g/L**) under GRN No. 897. Chr. Hansen intends to use 3-FL in oral and enteral tube feeding formula at up to **6.6 g/L** (**1.32 g/serving**). The clinical studies and scientific rationales to support the safety of 3-FL and 2'-FL for their intended uses in oral and enteral tube feeding formula have been described extensively in GRN No. 951 and GRN No. 897, respectively, which are incorporated by reference herein.

Enteral nutrition involves the administration of a liquid formula into the gastrointestinal tract as an exclusive or partial source of nutrition (Limketkai et al., 2019). These specialized formulas are intended for individuals who are malnourished or at risk of becoming malnourished (Kulick & Deen, 2011; Limketkai et al., 2019; NICE, 2017). They can be consumed orally, if the individual has a functional gastrointestinal tract and swallowing mechanisms (Kulick & Deen, 2011). If the gastrointestinal tract is functional but the individual is unable to safely swallow, then a feeding tube (e.g., nasogastric, nasoduodenal, gastrojejunostomy, jejunostomy) may be required (Kulick & Deen, 2011; Limketkai et al., 2019). While enteral nutrition plays an important role in providing nutritional support in a variety of care settings, there are some known complications that can arise from their use. Gastrointestinal intolerance (e.g., diarrhea) is one of the most common complications of enteral nutrition; it can be attributed to a number of potential factors such as concurrent use of antibiotics and other medications, underlying disease states, altered physiological responses, disrupted gastrointestinal microbiota and barrier function, and poor handling and preparation leading to contamination of the enteral product or delivery system (Boullata et al., 2017; Cara et al., 2021; Kulick & Deen, 2011). Accordingly, the provision of nutritional support through enteral nutrition is a complex and iterative process. Healthcare professionals determine the appropriate regimen (e.g., type of formula, route of administration, duration of nutritional support required) based on an initial assessment of the individual, followed by continual monitoring and reassessment to ensure tolerability and that nutritional goals are being met (Boullata et al., 2017; National Collaborating Centre for Acute Care, 2006).

Given the role of low-digestible carbohydrates (CHOs) in supporting gastrointestinal health (Institute of Medicine, 2005; Slavin, 2008), various oral and enteral tube feeding formulas containing low-digestible CHOs have been developed and are commercially available (ASPEN, 2020; Kulick & Deen, 2011). Enteral formula containing various types of low-digestible CHOs have been evaluated in a large number of randomized clinical trials and open-label studies involving preterm infants, children, healthy adults, bed-ridden elderly adults, and patients hospitalized for a variety of serious medical conditions. These studies have been tabulated in GRN No. 897, and again in GRN No. 951. Similar to the conclusions drawn in GRN No. 897, the notifier of GRN No. 951 stated that: "*The test articles include partially hydrolysed guar gum (PHGG), galactomannan, fructooligosaccharides (from scFOS to long-chain inulin), galacto-oligosaccharides, and GOS/FOS blends with ingestion levels often greater than 20 g/day and as high as 63 g/day. No adverse effects were reported in any study, suggesting that addition of not more than 4 g of 3-FL per serving of enteral feeds is safe.*" Although it was concluded in GRN No. 951 that addition of 3-FL into oral and enteral tube feeding formula at 4 g/serving (20 g/L) is safe, it should be noted that the use level of 3-FL in these products was subsequently reduced to 0.88 g/serving (4.4 g/L). Upon review of GRN No. 951 and the accompanying FDA correspondences, it seems the reduction was due to the fact the

notifier of GRN No. 951 decided to reduce the use level for 3-FL in infant formula from 2.0 g/L to 0.44 g/L, and thereby the use levels in all other intended food categories were also reduced by the same factor (4.5-fold). The reduction in use level for 3-FL in oral and enteral tube feeding formula in GRN No. 951 did not appear to be due to safety reasons, and the intended use level for Chr. Hansen's 3-FL in these products (1.32 g/serving, 6.6 g/L) is still well within the use level proposed originally in GRN No. 951 (4 g/serving, 20 g/L), and in GRN No. 897 for 2'-FL (20 g/L).

In addition to the clinical studies summarized in GRN No. 897[5] and GRN No. 951, the utility and safety of low-digestible CHOs in enteral nutrition have been the subject of several systematic reviews (e.g., Cara et al., 2021; Eleftheriadis & Davies, 2021; Elia et al., 2008; Heyland et al., 2021; Reis et al., 2018; Seifi et al., 2021; Zaman et al., 2015). Many of these reviews focus on clinical trials in which enteral formula containing low-digestible CHOs was administered to critically ill individuals under hospitalized settings. Nonetheless, the analyses conducted in systematic reviews and meta-analyses suggest the use of low-digestible carbohydrates in enteral nutrition generally appears to be safe and did not increase the risk of diarrhea amongst the critically ill (Cara et al., 2021; Eleftheriadis & Davies, 2021; Heyland et al., 2021; Reis et al., 2018; Seifi et al., 2021; Zaman et al., 2015). Similarly, in another systematic review that included studies involving healthy volunteers and patients in both hospital and community settings, it was concluded that enteral formulas containing low-digestible CHOs are well-tolerated (Elia et al., 2008). A total of 51 primary research studies were included in this review in which over 15 different types of low-digestible CHOs were administered, either on their own or as a combined mixture (e.g., soy polysaccharides, soy oligosaccharides, cellulose, gum arabic, FOS, oligofructose, inulin, resistant starch, partially hydrolyzed guar gum, pectin, psyllium, carboxymethylcellulose, oat fiber), with mean intakes ranging from approximately 7 to 40 g/day (Elia et al., 2008).

Although clinical studies have not been conducted with enteral formula containing 3-FL specifically, the available data support the judicious use of low-digestible CHOs in oral and enteral tube feeding formula, consistent with the conclusions drawn previously in GRN No. 897 and GRN No. 951. Similar to other fermentable short-chain oligosaccharides, 3-FL is resistant to digestive enzymes in the upper gastrointestinal tract, and instead reaches the colon where they can serve as growth substrates for bacterial fermentation, generating short-chain fatty acids and organic acids (e.g., lactate) (Kong et al., 2021; Li et al., 2022; Wiese et al., 2018). As mentioned above, enteral nutrition does not follow a one-size-fits-all approach. In some instances, oral and enteral tube feeding formulas containing low-digestible CHOs may not be suitable. As discussed in GRN Nos. 897 and 951, it has been suggested by Tarleton et al. (2013) that although the addition of low-digestible CHOs to enteral formulas is meant to normalize bowel function and improve feeding tolerance, the presence of certain comorbidities may contraindicate their use. In particular, the authors noted two specific medical conditions (*i.e.*, patients at high risk for bowel ischemia or severe dysmotility) for whom the addition of low-digestible CHOs into enteral feeds may not be well tolerated. Nonetheless, these are both easily observable conditions, and it is likely that the healthcare professionals overseeing the administration of partial or total enteral nutrition would be aware of the patient's status and thereby avoid the use of formulas containing low-digestible CHOs.

[5] With respect to the clinical studies identified in GRN No. 897, the notifier had stated that: "*While no claim is made that this survey of the literature is exhaustive, it is not selective in choosing only supportive research*."

There are many different types of oral and enteral tube feeding formulas on the market, and selection of the appropriate formula for a given individual depends on numerous factors such as age, gastrointestinal function, health status/medical history, nutritional status, and fluid status (ASPEN, 2020; Boullata et al., 2017; Limketkai et al., 2019; NICE, 2017). The manufacturers of oral and enteral tube feeding formulas will ensure that the products are formulated in a manner that minimizes the potential for adverse events in the specific population for whom the products are intended. This would include consideration of whether low-digestible CHOs should be included, and if so, which type(s) of low-digestible CHOs are the most appropriate for inclusion. Healthcare professionals also have an important role in ensuring the assigned enteral nutrition regimen is appropriate and well tolerated by the individual requiring such products. As it is expected that oral and enteral tube feeding formula containing 3-FL will be given only to individuals for whom such products are suitably indicated, the conclusion that was made in GRN No. 897 is also applicable for Chr. Hansen's 3-FL: "*Research to date bears out the belief that risks of adverse effects from judicious addition of low-digestible CHO to enteral formula, while probably not zero, are well within the GRAS standard of relative certainty of no harm*."

6.5 Conclusion of GRAS Status

The safety of Chr. Hansen's 3-FL under its intended conditions of use as a food ingredient is supported by the following:

- The GRAS status of Chr. Hansen's 3-FL for use in non-exempt term infant formula at up to 0.44 g/L (as consumed) has been previously notified to the U.S. FDA and filed with "no questions" under GRN No. 925.

- As described in GRN No. 925, 3-FL manufactured by Chr. Hansen is chemically and structurally identical to 3-FL in human milk. The production process is conducted in accordance with cGMP, and strict manufacturing controls are in place to ensure quality of the finished product. The finished material is a spray-dried powder containing ≥90% 3-FL, with the remaining components comprising small amounts of residual carbohydrate by-products, ash, and moisture. The production organism, *E. coli* BL21(DE3) strain JBT-3FL, is genetically engineered to express the biosynthetic pathways needed for 3-FL production. The production strain is safe for use; it is non-pathogenic and non-toxigenic, and is 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 JBT-3FL strain remains in the finished 3-FL material.

- HMOs, including 3-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 in the feces. Since Chr. Hansen's 3-FL is structurally equivalent to its naturally occurring counterpart in human milk, it is expected to undergo the same ADME processes as those in infants consuming naturally occurring 3-FL in human milk.

- Chr. Hansen's 3-FL, as part of a mixture containing five HMOs, has been evaluated in preclinical toxicological studies, including mutagenicity/genotoxicity assays (bacterial reverse mutation assay, *in vitro* micronucleus test) and a 90-day oral toxicity study in rats (Parschat et al., 2020), as well as a 21-day tolerance study in neonatal piglets (Hanlon, 2020). Additionally, preclinical

toxicology studies have been conducted with 3-FL preparations produced by other manufacturers that are compositionally and quantitatively similar to Chr. Hansen's 3-FL ingredient (EFSA NDA Panel, 2021; Phipps et al., 2022; Pitt et al., 2019). These studies further demonstrate that 3-FL does not pose any toxicological concerns.

- In addition to the preclinical toxicology data, two randomized, controlled, clinical studies have further demonstrated the safety and tolerability of infant formulas containing 3-FL at 0.75 g/L in term infants (Abbott Nutrition, 2021; Parschat et al., 2021).

- The intended uses for Chr. Hansen's 3-FL in additional food categories, as described in this GRAS notice, are similar to those that have been notified for 3-FL under GRN No. 951, which has been filed by the FDA with "no questions". Although the intended use levels for 3-FL in infant formula (0.9 g/L) and toddler formula (1.2 g/L) are now higher than those indicated in GRN No. 925 and GRN No. 951 (0.44 g/L), the use levels continue to be within concentrations reported for 3-FL in human breastmilk (Conze et al., 2022; EFSA NDA Panel, 2021; Soyyılmaz et al., 2021; Thurl et al., 2017). Accordingly, the 90th percentile EDI of 3-FL in infants (0 to 6 months: 199 mg/kg bw/day; 7 to <12 months: 187 mg/kg bw/day) and toddlers age 1 to 3 years old (91 mg/kg bw/day) are comparable to those of infants who are fed human milk (upwards of 251 mg/kg bw/day). The 90th percentile EDIs for 3-FL by older population groups (18 to 35 mg/kg bw/day) are also well within the estimated intake level for 3-FL by infants consuming this HMO through its natural occurrence in human milk.

From the data and information presented herein, Chr. Hansen concludes their 3-FL produced with genetically engineered *E. coli* BL21(DE3) strain JBT-3FL is GRAS for its intended uses as described herein, based on scientific procedures. All pivotal data and information used to establish the safety of Chr. Hansen's 3-FL under its intended conditions of use are "generally available" (*i.e.*, in the public domain). Moreover, this GRAS conclusion was reached in concert with the views of a panel of experts (the GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of food and food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Dr. George C. Fahey, Jr. (Professor Emeritus, University of Illinois), Dr. Michael W. Pariza (Professor Emeritus, University of Wisconsin-Madison), and Dr. Madhusudan G. Soni (Soni & Associates Inc.). A copy of the GRAS Panel Consensus Statement is provided in Appendix A.

7. List of Supporting Data and Information

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Appendix A:
GRAS Panel Consensus Statement

GRAS Panel Statement for the Intended Uses of Chr. Hansen's 3-Fucosyllactose in Infant Formula, Conventional Foods, and Oral/Enteral Tube Feeding Formulas

May 2022

Introduction

A panel of independent experts ("the GRAS Panel"), qualified by scientific training and experience to evaluate the safety of food and food ingredients, was convened by Chr. Hansen A/S ("Chr. Hansen") to evaluate the Generally Recognized as Safe (GRAS) status of 3-fucosyllactose (3-FL), produced with genetically engineered *Escherichia coli* BL21(DE3) strain JBT-3FL, for its intended uses in infant formula and other foods as described in Table 1. The GRAS Panel consisted of the following qualified scientific experts: Dr. George C. Fahey, Jr. (Professor Emeritus, University of Illinois), Dr. Michael W. Pariza (Professor Emeritus, University of Wisconsin-Madison), and Dr. Madhusudan G. Soni (Soni & Associates Inc.).

A comprehensive review of the scientific literature and other information pertinent to the safety evaluation of 3-FL under its intended conditions of use was conducted by Chr. Hansen and compiled into a dossier titled: "*Documentation to Support the Generally Recognized as Safe (GRAS) Status of 3-Fucosyllactose (3-FL) for its Intended Uses in Infant Formula, Conventional Foods, and Oral/Enteral Tube Feeding Formulas*" (dated May 5, 2022). The GRAS Panel, independently and collectively, evaluated the materials provided by Chr. Hansen and other information deemed appropriate or necessary. Following their critical evaluation, the GRAS Panel unanimously concluded that 3-FL produced with *E. coli* BL21(DE3) strain JBT-3FL is GRAS for its intended uses in infant formula and other foods, based on scientific procedures. A summary of the basis supporting the GRAS Panel's conclusion is presented below.

Summary and Basis for GRAS

Human breastmilk contains a unique fraction of structurally diverse non-digestible carbohydrates known as human milk oligosaccharides (HMOs), which represent the third largest solid component in breastmilk after lactose and lipids (Bode, 2012, 2019). Although over 200 different HMO structures have been identified, a subset of just 15 to 20 HMOs account for the majority of the total oligosaccharide fraction of human milk, with fucosyllactoses (including 3-FL) being amongst the most abundant HMOs present (EFSA NDA Panel, 2014; Soyyılmaz et al., 2021). 3-FL is a fucosylated, neutral trisaccharide consisting of L-fucose, D-galactose, and D-glucose. As part of ongoing efforts to bring the composition of formula products closer to that of human milk, manufactured versions of purified HMOs, including 3-FL, have been developed as ingredients in infant formula.

Chr. Hansen has previously notified the United States (U.S.) Food and Drug Administration (FDA) of their conclusion that 3-FL produced with genetically engineered *E. coli* BL21(DE3) strain JBT-3FL is GRAS for its intended use as an ingredient in cow milk-based, non-exempt infant formula for term infants at a level of 0.44 g/L of formula, as consumed[1]. This GRAS notice was filed by the FDA with "no questions" under GRN No. 925. Chr. Hansen now intends to increase the use level of 3-FL in non-exempt infant formula (including cow-milk-, soy-milk-, and partially hydrolyzed protein-based formulas) to 0.9 g/L, as consumed. Furthermore, Chr. Hansen's 3-FL ingredient is intended for use in additional food categories, as listed in

[1] This 3-FL ingredient was initially developed by Jennewein Biotechnology GmbH, which was acquired by Chr. Hansen A/S in 2020.

Table 1, which are similar to those that have been concluded GRAS for 3-FL in a notice submitted by Danisco USA, Inc. This GRAS notice was also filed with "no questions" by the FDA under GRN No. 951.

Chr. Hansen's 3-FL is produced by fermentation with *E. coli* BL21(DE3) strain JBT-3FL as a processing aid. The 3-FL ingredient is produced using the same manufacturing processes, and there have been no changes to its composition or specifications since the completion of GRN No. 925. A series of genetic modifications are introduced into the *E. coli* BL21(DE3) host organism to allow for the biosynthesis of 3-FL using lactose as a substrate. The JBT-3FL production strain is safe for use; it is non-pathogenic and non-toxigenic, and is not capable of DNA transfer to other organisms. The fermentation process for 3-FL is conducted in a contained, sterile environment. Batch fermentation is performed in a minimal medium containing a simple, pure carbon source (e.g., glucose, sucrose, glycerol) and the lactose substrate. During fermentation, 3-FL is produced and secreted into the culture medium by the JBT-3FL production strain. Once the fermentation step is completed, the culture medium is separated from the microbial biomass, and 3-FL is further purified from the culture medium through a series of filtration, ion exchange, electrodialysis, and decolorization steps. The resulting 3-FL concentrate is then spray-dried into a powdered product. The production process is conducted in accordance with current Good Manufacturing Practice (cGMP), and strict manufacturing controls are in place to ensure quality of the finished product. The finished material is a spray-dried powder containing ≥90% 3-FL, with the remaining components comprising small amounts of residual carbohydrate by-products, ash, and moisture. Analytical data demonstrate the 3-FL manufactured by Chr. Hansen is chemically and structurally identical to 3-FL in human milk.

HMOs, including 3-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 in the feces. As Chr. Hansen's 3-FL is structurally equivalent to its naturally occurring counterpart in human milk, it is expected to undergo the same metabolic processes as those in infants consuming naturally occurring 3-FL in human milk. As described in GRN No. 925, comprehensive toxicological studies have been conducted with Chr. Hansen's 3-FL, which was evaluated as part of a mixture called "5HMO-Mix". No evidence of mutagenicity/genotoxicity were observed in a bacterial reverse mutation assay and *in vitro* micronucleus test (Parschat et al., 2020). Additionally, no adverse effects were observed in 90-day dietary toxicity study conducted with 5HMO-Mix in rats, with the no-observed-adverse-effect level (NOAEL) concluded as 5.67 g/kg body weight (bw)/day for males and 6.97 g/kg bw/day for females. Considering the 5HMO-Mix test article contains 16.0% dry weight of 3-FL, this corresponds to intakes of 0.91 g 3-FL/kg bw/day for males and 1.12 g 3-FL/kg bw/day for females (Parschat et al., 2020). In a 21-day neonatal piglet study, milk replacer containing 5HMO-Mix at up to 8.0 g/L (providing approximately 0.37 g 3-FL/kg bw/day) was concluded to be safe and well-tolerated (Hanlon, 2020). Moreover, no adverse effects were observed in preclinical toxicology studies conducted with 3-FL preparations produced by other manufacturers that are compositionally and quantitatively similar to Chr. Hansen's 3-FL ingredient (EFSA NDA Panel, 2021; Phipps et al., 2022; Pitt et al., 2019).

Since the filing of GRN No. 925 and GRN No. 951, two randomized, controlled, clinical studies have further demonstrated the safety and tolerability of infant formulas containing 3-FL (NCT03513744, NCT04105686). In one published study, 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 (Parschat et al., 2021).

The 5HMO-Mix provided 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. There were no statistically significant differences in weight, length, or head circumference gain between the two formula groups, and formula supplemented with 5HMO-Mix was considered non-inferior to the control formula with respect to mean daily body weight gain. The total incidence of adverse events (AEs) and serious AEs were comparable across all three groups. Based on these results, the study authors concluded the addition of 5HMO-Mix into infant formula is safe and well-tolerated. Similarly, in a second clinical study, consumption of an infant formula containing 5HMO-Mix (including 0.75 g/L of 3-FL) for 16 weeks was safe, well-tolerated, and supported normal growth by healthy term infants (Abbott Nutrition, 2021).

The intended uses for Chr. Hansen's 3-FL in additional food categories, as described herein, are similar to those that have been notified as GRAS for 3-FL under GRN No. 951, which has been filed by the FDA with "no questions". Although the intended use levels for 3-FL in infant formula (0.9 g/L) and toddler formula (1.2 g/L) are now higher than those indicated in GRN No. 925 and GRN No. 951 (0.44 g/L), the use level continues to be within concentrations reported for 3-FL in human breastmilk (Conze et al., 2022; EFSA NDA Panel, 2021; Soyyılmaz et al., 2021; Thurl et al., 2017). Accordingly, the 90th percentile estimated daily intake (EDI) of 3-FL in infants (0 to 6 months: 199 mg/kg bw/day; 7 to <12 months: 187 mg/kg bw/day) and toddlers age 1 to 3 years old (91 mg/kg bw/day) are comparable to those of infants who are fed human milk (upwards of 251 mg/kg bw/day). The 90th percentile EDIs for 3-FL by older population groups (18 to 35 mg/kg bw/day) is also well within the estimated intake level for 3-FL by infants consuming this HMO through its natural occurrence in human milk.

In addition to its intended uses in infant formula and conventional foods, 3-FL has been previously concluded GRAS for its intended use in oral and enteral tube feeding formula at up to 4.4 g/L (0.88 g/serving) under GRN No. 951. Notably, 2'-fucosyllactose (which is a structurally-related isomer of 3-FL) has also been concluded GRAS for use in oral and enteral tube feeding formula at up to 20 g/L under GRN No. 897. Chr. Hansen intends to use 3-FL in oral and enteral tube feeding formula at up to 6.6 g/L (1.32 g/serving). Clinical studies have not been conducted with enteral formula containing 3-FL or other HMOs to date. However, various oral and enteral tube feeding formulas containing low-digestible carbohydrates (CHOs) have been developed and are commercially available (ASPEN, 2020; Kulick & Deen, 2011). A number of clinical studies have also examined enteral formula containing various types of low-digestible CHOs, which have been summarized in GRN No. 897 and GRN No. 951, as well as various systematic reviews (e.g., Cara et al., 2021; Eleftheriadis & Davies, 2021; Elia et al., 2008; Heyland et al., 2021; Reis et al., 2018; Seifi et al., 2021; Zaman et al., 2015). As discussed in GRN Nos. 897 and 951, it has been suggested that although the addition of low-digestible CHOs to enteral formulas is meant to normalize bowel function and improve feeding tolerance, their use may be contraindicated for some individuals, such as those with a high risk for bowel ischemia or severe dysmotility (Tarleton et al., 2013). Nonetheless, it is expected that oral and enteral tube feeding formula containing 3-FL will be given only to individuals for whom such products are suitably indicated. Accordingly, the conclusion that was made in GRN No. 897 is also considered applicable for Chr. Hansen's 3-FL: "*Research to date bears out the belief that risks of adverse effects from judicious addition of low-digestible CHO to enteral formula, while probably not zero, are well within the GRAS standard of relative certainty of no harm*."

Table 1 Intended Food Uses and Use Levels for 3-FL in the United States

Food Category (21 CFR §170.3)	Food Uses[a,b]	Use Levels (g/L or g/kg)
Infant and Toddler Foods	Non-exempt term infant formula (0-12 months)	**0.9[c] (as consumed)**
	Toddler formula (1-3 years)	**1.2[c] (as consumed)**
	Other drinks for young children, incl. yogurt and juice beverages identified as "baby drinks"	0.44
	Hot cereals (dry and RTE)	4.4
	Baby crackers, pretzels, cookies, and snack items	4.4
Beverages and Beverage Bases	Enhanced and fortified waters (incl. flavored and carbonated waters)	0.26
	Non-milk-based meal replacement drinks	**4.0[d]**
	Sports, isotonic, and energy drinks	**0.3[e]**
Breakfast Cereals	Hot Breakfast Cereals (*e.g.*, oatmeal, grits), instant and RTE	6.8
	RTE breakfast cereals	8.8
Dairy Product Analogs	Milk substitutes such as soy milk and imitation milks	0.26
	Non-dairy yogurt	2.64
Grain Products and Pastas	Cereal and granola bars incl. energy, protein, and meal replacement bars	6.6
Milk Products	Fermented milk	0.26
	Flavored milk (incl. RTD and powder mix)	0.26
	Milk-based meal replacement beverages	**4.0[d]**
	Smoothies (dairy and non-dairy)	1.1
	Yogurt	2.64
Processed Fruits and Fruit Juices	Fruit flavored drinks and ades	0.26
	Fruit juices and nectars	0.26
Processed Vegetables and Vegetable Juices	Vegetable juices	0.26
Soft Candy	**Gummy candies**	**8.8**
Foods for Special Dietary Uses	Oral and enteral tube feeding formulas[f]	**6.6[g] (final product, as consumed)**

Abbreviation(s): 3-FL = 3-fucosyllactose; CFR = Code of Federal Regulations; GRAS = Generally Recognized as Safe; incl. = including; RTD = ready-to-drink; RTE = ready-to-eat.

[a] 3-FL is intended for use in unstandardized products where standards of identity, as established under 21 CFR §130 to 169, do not permit its addition in standardized products.

[b] Additional food categories and/or use levels that have not been previously concluded as GRAS and notified to the U.S. FDA are **bolded**.

[c] 3-FL was previously concluded to be GRAS for use in infant formula (GRN 925, 951) and toddler formula (GRN 951) at a use level of 0.44 g/L.

[d] 3-FL was previously concluded to be GRAS for use in meal replacement beverage at a use level of 1.1 g/L (GRN 951).

[e] 3-FL was previously concluded to be GRAS for use in sports, isotonic, and energy drinks at a level of 0.26 g/L (GRN 951).

[f] Foods for special dietary use were assessed separately from the intended food uses of 3-FL in conventional foods, as they are intended for supplying a particular dietary need. Intake of 3-FL from foods for special dietary use is, therefore, not expected to be cumulative to other dietary sources.

[g] 3-FL was previously concluded to be GRAS for use in foods for special dietary uses at a use level of 4.4 g/L (GRN 951).

Conclusions

We, the undersigned members of the GRAS Panel, have critically evaluated the data and information summarized above, and unanimously conclude that 3-fucosyllactose produced by fermentation with *E. coli* BL21(DE3) strain JBT-3FL is safe for its intended uses as an ingredient in infant formula and other foods as described herein.

We further unanimously conclude that 3-fucosyllactose produced by fermentation with *E. coli* BL21(DE3) strain JBT-3FL, meeting food-grade specifications and manufactured consistent with current Good Manufacturing Practice, is GRAS for its intended conditions of use, based on scientific procedures.

It is our opinion that other qualified experts reviewing the same publicly available information would concur with these conclusions.

DocuSigned by:
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18 May 2022 | 09:47 CDT

George C. Fahey, Jr., Ph.D.
Professor Emeritus
University of Illinois
Urbana, Illinois

Date

DocuSigned by:
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18 May 2022 | 18:08 CEST

Michael W. Pariza, Ph.D.
Professor Emeritus
University of Wisconsin-Madison
Madison, Wisconsin

Date

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18 May 2022 | 15:47 CEST

Madhusudan G. Soni, Ph.D., F.A.C.N., F.A.T.S.
Soni & Associates Inc.

Date

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From: Manki Ho
To: Morissette, Rachel
Subject: [EXTERNAL] RE: question for GRN 001099
Date: Wednesday, July 26, 2023 1:22:31 PM
Attachments: image001.png
image002.png
image003.png
image004.png
image005.png
image006.png

Dear Rachel,

Regarding your question below, Chr. Hansen confirms the 3-FL ingredient is intended for use in oral and enteral tube feeding formulas for consumers ≥ 11 years of age.

We hope this addresses your query. Please let us know if you need anything else.

With kind regards,
Manki

Manki Ho, Ph.D.
Principal Regulatory Affairs Specialist

Email: camaho@chr-hansen.com
Mobile: +1-647-928-5735

My preferred pronouns: she/her/hers

www.chr-hansen.com – LinkedIn – Twitter – Facebook

From: Morissette, Rachel <Rachel.Morissette@fda.hhs.gov>
Sent: Tuesday, July 25, 2023 4:05 PM
To: Manki Ho <CAMAHO@chr-hansen.com>
Subject: question for GRN 001099

Hi Manki,

Please see below a question for GRN 001099. We are drafting your response letter now, so please provide a response within 5 business days if possible.

Page 31 of the notice states: "The clinical studies and scientific rationales to support the safety of 3-FL and 2'-FL for their intended uses in oral and enteral tube feeding formula have been described extensively in GRN No. 951 and GRN No. 897, respectively, which are incorporated by reference herein." We note that both referenced GRNs specify the intended use in oral and enteral tube feeding for consumers ≥ 11 years of age. Since Chr. Hansen did not specify the age group in GRN 001099, please confirm that the intended use in oral and enteral tube feeding is identical to that in GRNs 000897 and 000951.

Best regards,

Rachel

Rachel Morissette, Ph.D.
Regulatory Review Scientist/Biologist

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