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GRN 000735

September 29, 2017

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety (HFS-255)
5001 Campus Drive
College Park, MD 20740-3835
Attention: Dr. Paulette Gaynor
Re: GRAS Notification—2'-Fucosyllactose

Dear Dr. Gaynor:

GRAS Associates, LLC, acting as the agent for Glycosyn, LLC (Woburn, MA) and FrieslandCampina Domo B.V. (The Netherlands) is submitting for FDA review Form 3667 and the enclosed CD, free of viruses, containing a GRAS notification for *2'-Fucosyllactose* produced by microbial fermentation. Along with Glycosyn and FrieslandCampina's determination of safety, an Expert Panel of qualified persons was assembled to assess the composite safety information of the subject substance with the intended use as an ingredient in infant formulas, conventional foods, and medical foods at levels ranging from 0.24 to 4.0 grams 2'-fucosyllactose per serving. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email.

We look forward to your feedback.

Sincerely,

(b) (6)

Katrina V. Emmel, Ph.D.
Senior Scientist/Associate
GRAS Associates, LLC
27499 Riverview Center Blvd., Suite 212
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Enclosure: GRAS Notification for Glycosyn and FrieslandCampina –*2'-Fucosyllactose*

DEPARTMENT OF HEALTH AND HUMAN SERVICES
Food and Drug Administration

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

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

FDA USE ONLY

GRN NUMBER	DATE OF RECEIPT
000735	RECEIVED OCT 2 2017 OFFICE OF FOOD ADDITIVE SAFETY
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

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

SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION

1. Type of Submission *(Check one)*

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

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

3 Most recent presubmission meeting *(if any)* with FDA on the subject substance *(yyyy/mm/dd)*: 2017/08/31

☐
☐

SECTION B – INFORMATION ABOUT THE NOTIFIER

1a. Notifier

Name of Contact Person	Position or Title
Howard Newburg	Chief Executive Officer

Organization *(if applicable)*: Glycosyn, LLC

Mailing Address *(number and street)*: 890 Winter Street, Suite 208

City	State or Province	Zip Code/Postal Code	Country
Waltham	Massachusetts	02451	United States of America

Telephone Number	Fax Number	E-Mail Address
781-884-4101	781-884-4151	hnewburg@glycosynllc.com

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

Name of Contact Person	Position or Title
Katrina Emmel	Senior Scientist/Associate

Organization *(if applicable)*: GRAS Associates, LLC

Mailing Address *(number and street)*: 27499 Riverview Center Blvd

City	State or Province	Zip Code/Postal Code	Country
Bonita Springs	Florida	34134	United States of America

Telephone Number	Fax Number	E-Mail Address
239-444-1724	239-444-1723	emmel@gras-associates.com

SECTION C – GENERAL ADMINISTRATIVE INFORMATION

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

2'-Fucosyllactose; 2'-FL

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

☐ Electronic Submission Gateway

☒ Electronic files on physical media

☐ Paper

If applicable give number and type of physical media

CD

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

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

☐

☐

☐

☐

☐

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

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

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

☐ Yes *(Proceed to Item 8*

☒ No *(Proceed to Section D)*

☐

☐

☐

☐

☐

SECTION D – INTENDED USE

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

2'-Fucosyllactose is intended for use in a number of conventional foods and medical foods, as well as conventional infant formula for full term infants. No uses in pre-term infants are proposed at this time. Proposed use levels range from 0.24-4 g 2'-fucosyllactose per serving.

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

(Check one)

☐ Yes ☒ No

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

(Check one)

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

SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE

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

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

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

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

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

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

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

Other Information

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

☒ Yes ☐ No

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

☒ Yes ☐ No

SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS

1. The undersigned is informing FDA that Glycosyn, LLC and FrieslandCampina Domo B.V.
(name of notifier)

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

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

2. Glycosyn, LLC and FrieslandCampina Domo B.V. agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them;
(name of notifier)

agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

Glycosyn, LLC, 6H Gill St., Woburn, MA, 01801
(address of notifier or other location)

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

3. Signature of Responsible Official, A(b) (6)	Printed Name and Title	Date (mm/dd/yyyy)
	Katrina Emmel, Ph.D. Senior Scientist/Associate	09/29/2017

SECTION G – LIST OF ATTACHMENTS

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

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	Volume II containing appendices 1-12	
	Appendix 6 qPCR 2'-fucosyllactose.pdf	
	Appendix 8 U.S. Intakes Report 2'-FL.pdf	
	Appendix 9 Final study report 14-day DRF 2'-FL toxicology test. pdf	
	Appendix 10 Sub-Chronic (13-week) Oral Toxicity Study.pdf	

OMB Statement: Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services,Food and Drug Administration, Office of Chief Information Officer, PRAStaff@fda.hhs.gov. (Please do NOT return the form to this address.). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

GRAS ASSOCIATES, LLC

GRAS Notification

of

Purified 2'-Fucosyllactose (2'-FL)

Food Usage Conditions for General Recognition of Safety

on behalf of

Glycosyn, LLC
Woburn, MA

and

FrieslandCampina Domo B.V.
Amersfoort, The Netherlands

Volume 1 of 2

9/29/17

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FOREWORD

At the request of Glycosyn, LLC ("Glycosyn") and FrieslandCampina Domo B.V. ("FrieslandCampina"), GRAS Associates, LLC ("GA") has reviewed an independent GRAS evaluation of FrieslandCampina's purified 2'-fucosyllactose (2'-FL). The purpose of the evaluation is to confirm the GRAS conclusion that the intended food uses of purified 2'-fucosyllactose (2'-FL) as a food ingredient as described in Part 3 are generally recognized as safe, i.e., GRAS, under the intended conditions of use. In addition, Glycosyn and FrieslandCampina have asked that GRAS Associates act as agent for the submission of this GRAS notification.

Glycosyn and FrieslandCampina based their GRAS assessment on a large body of information that addressed the safety/toxicity of 2'-FL, history of use of 2'-FL and similar compounds, and compositional details, specifications, and method of preparation of the subject ingredient.

Safety/toxicity studies performed with animals and human clinical trials were noted to have value. The composite safety/toxicity studies, in concert with dietary exposure information, ultimately provide the specific scientific foundation for the GRAS conclusion.

In addition to the product specifications, chemical properties, manufacturing, and safety-related information, Glycosyn and FrieslandCampina also provided consumption/exposure information, along with other related documentation. This was augmented with an independent search of the scientific and regulatory literature extending through July 13th, 2017. A GRAS assessment based primarily on the composite safety information, i.e., based on scientific procedures, was undertaken by Glycosyn and FrieslandCampina, followed by an Expert Panel review coordinated by GRAS Associates. Those references that were deemed pertinent to this review are listed in Part 7.

PART 1. SIGNED STATEMENTS AND CERTIFICATION

A. Claim of Exclusion from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR 170.36(c)(1)[1]

Glycosyn and FrieslandCampina have concluded that their purified 2'-fucosyllactose product, referred to as 2'-FL, and which meets the specifications described below, is Generally Recognized As Safe (GRAS) in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. This determination was supported by a review by an appropriately convened panel of experts who are qualified by scientific training and experience. The GRAS determination is based on scientific procedures as described in this notice. The evaluation accurately reflects the intended conditions of food use for the designated 2'-fucosyllactose product.

[1] See 81 FR 54960, 17 August 2016. Accessible at: https://www.gpo.gov/fdsys/pkg/FR-2016-08-17/pdf/2016-19164.pdf (Accessed 7/4/17).

Signed:

(b) (6)

Agent for Glycosyn and FrieslandCampina

Steven Overgaard
President
GRAS Associates, LLC
27499 Riverview Center Blvd.
Suite 212
Bonita Springs, FL 34134

Date: 9/29/17

B. Names and Addresses of Responsible Parties

Glycosyn, LLC
6H Gill Street
Woburn, MA
01801

FrieslandCampina Domo B.V.
Stationsplein 4,3818 LE Amersfoort
P.O. Box 1551, 3800 BN Amersfoort
The Netherlands

As the Responsible Parties, Glycosyn, LLC and FrieslandCampina Domo accept responsibility for the GRAS conclusion that has been made for their purified 2'-fucosyllactose, as described in the subject safety evaluation; consequently, their purified 2'-fucosyllactose having purity no less than 90% 2'-FL, which meet the conditions described herein, are not subject to premarket approval requirements for food ingredients.

C. Common Name and Identity of Subject Substance

The common name of the ingredient to be used on food labels is 2'-fucosyllactose.

D. Conditions of Intended Use in Food

2'-Fucosyllactose is intended for use as an ingredient in infant formulas, conventional foods, and medical foods in the United States at the use levels described in Part 3.

E. Basis for GRAS Conclusion

Pursuant to 21 CFR 170.30(a) and (b), Glycosyn and FrieslandCampina's purified 2'-fucosyllactose preparation (> 90% 2'-FL) has been concluded to be GRAS on the basis of scientific procedures as discussed in the detailed description provided below.

Purified 2'-fucosyllactose is not subject to premarket approval requirements of the FD&C Act based on Glycosyn and FrieslandCampina's conclusion that the substance is GRAS under the conditions of its intended food use.

Glycosyn, FrieslandCampina, and GRAS Associates certify, to the best of our knowledge, that this GRAS notice is a complete, representative, and balanced assessment that includes all relevant information, both favorable and unfavorable, available and pertinent to the evaluation of safety and GRAS status of purified 2'-fucosyllactose.

F. Availability of Information

The data and information that serve as the basis for this GRAS Notice will be maintained at the offices of Glycosyn, LLC, 6H Gill Street, Woburn, MA, and at the offices of FrieslandCampina Domo B.V., Stationsplein 4,3818 LE Amersfoort, P.O. Box 1551, 3800 BN Amersfoort, The Netherlands, and will be made available during customary business hours.

Glycosyn, FrieslandCampina, and GRAS Associates, LLC certify that no data or information contained herein are exempt from disclosure under the Freedom of Information Act (FOIA). Appendices 9-12 of this notice are full study reports of unpublished toxicological studies that confirm publicly available safety-related data for 2'-FL. These studies were considered corroborative by Glycosyn, FrieslandCampina, and the Expert Panel in this GRAS conclusion.

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

A. Chemical Identity of Ingredient

2'-FL is a trisaccharide that is naturally occurring in human breast milk.

Chemical name: α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose.

Synonyms: 2'-*O*-fucosyllactose; 2'-*O*-L-fucosyl-D-lactose; fucosyl-α-1,2-galactosyl-β-1,4-glucose; Fuc-α-(1→2)-Gal-β-(1→4)-Glc; 2'-FL.

Chemical formula: $C_{18}H_{32}O_{15}$

Molecular weight: 488.44 daltons

CAS Number: 41263-94-9

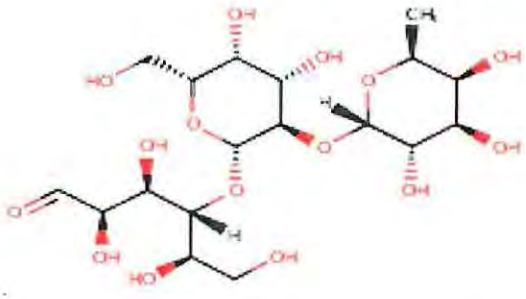
1. Chemical Structure of Purified 2'-Fucosyllactose

2'-FL is a trisaccharide composed of L-fucose, D-galactose, and D-glucose. The monosaccharide L-fucose is linked to the disaccharide D-lactose by an α-(1→2) bond. Structural representations from various reference sources are depicted in Figure 1.

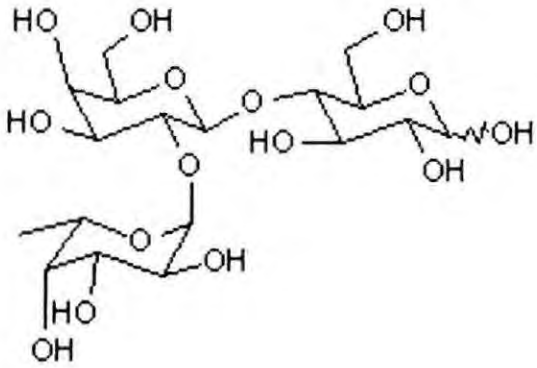
The primary constituent of the subject ingredient is 2'-FL (> 90%), with minor concentrations (maximum 3% each) of related sugars, including lactose, allo-lactose, glucose, galactose, and fucose.

Figure 1. Chemical Structure of 2'-Fucosyllactose

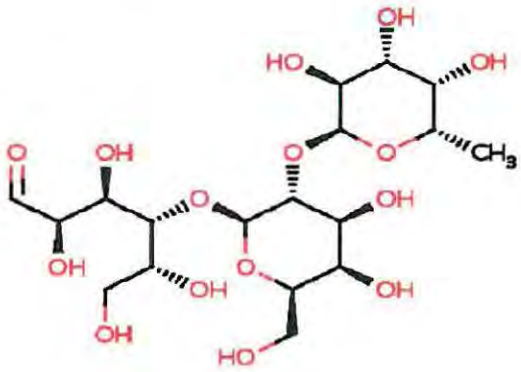
a. ChemIDplus (2016a)



b. Carbosynth (2016)



c. HMDB (2016)



2. Background on the *E. coli* K12 E638 Host Strain and E997 Fermentation Production Strain

Many publications have described using bacterial vectors, where recombinant genes for fucosyltransferase (FucT) and guanosine 5'-diphospho-b-L-fucose (GDP-L-fucose) synthases are expressed to produce 2'-FL. A short summary of published studies is provided below.

Albermann et al. (2001) reported using an *in vitro* method to produce 2'-fucosyllactose using recombinant fucosyltransferase (FucT2) from *Helicobacter pylori (H. pylori)* to transfer enzymatically-synthesized GDP-L-fucose to lactose (Abstract only).

Engineered *Escherichia coli* (*E. coli)* strains BL21star(DE3) and JM109(DE3) were investigated for 2'-FL production using the FucT2 *H. pylori* gene. Whole cell biosynthesis of 2'-FL was observed; however, the authors noted an experimental yield of only 20% of the theoretical yield (Lee et al., 2012).

Baumgartner et al. (2013) investigated using recombinant *in vivo* biosynthesis to produce 2'-FL in genetically engineered *E. coli* JM109. Recombinant genes for *de novo* GDP-L-fucose synthesis were integrated into the *E. coli* chromosome, as well as *futC* from *H. pylori* and/or *fkp* from *Bacteroides fragilis,* to create plasmid-free *E. coli* strains capable of synthesizing 2'-FL from lactose and glycerol.

In 2014, Engels and Elling identified WbgL, a bacterial α-1,2-fucosyltransferase encoded in the *E. coli* O126 genome. The authors demonstrated that recombinant strains of *E. coli* BL21(DE3) and *E. coli* JM109(DE3) were able to express the active enzyme. Subsequently, Engels and Elling used the recombinant enzyme to synthesize 2'-FL from GDP-L-fucose in both two-step and sequential reactions.

More recently, Chin et al. (2015) reported using bioengineered *E. coli* BL21star (DE3) to express GDP-L-fucose synthesis enzymes and α-1,2-fucosyltransferease (FucT2) from *H. pylori* to produce 2'-FL (Abstract only).

The background host organism Glycosyn and FrieslandCampina use in their 2'-FL process is non-pathogenic *E. coli* strain K12. K12 is the standard "workhorse" organism used for most recombinant DNA work in laboratories worldwide. It has a defective cell envelope that renders it incapable of colonizing or surviving in the human gut.

Glycosyn and FrieslandCampina's specific production *E. coli* host is derived directly from strain "GI724," which has been well defined. The strain is in the W3110 lineage of *E. coli* K12 (Bachmann, 1972; Hayashi et al., 2006). Strain RB791 passed through Mark Ptashne's laboratory at Harvard University in the early 1980s (Brent and Ptashne, 1981), and then strain GI724 passed through Genetics Institute (LaVallie et al., 1993). A close relative of GI724 was developed for, and is currently used in, the commercial manufacture of the FDA-approved biopharmaceutical injectable protein Oprelvekin, a recombinant human interleukin 11, and the active ingredient in "Neumega" (Genetics Institute, 2002). Glycosyn obtained *E. coli* strain GI724 as the parent organism from the American Type Culture Collection (ATCC® number 55151).

GI724 is a prototroph, capable of growth on defined media containing only inorganic salts and a suitable carbon source. Complete deoxyribonucleic acid (DNA) sequencing of GI724 performed by Glycosyn confirms the strain to be completely free of any virulence genes, and very closely related

to both the MG1655 and W3110 lineages of *E. coli* K12 (Bachmann, 1972; Blattner et al., 1997; Schultz, 2008).

Several specific genetic manipulations were performed on *E. coli* strain GI724 to develop a new background host for the commercial production of fucosylated oligosaccharides. These genetic changes are summarized in Table 1, and the newly engineered host carries the strain designation "E638."

Table 1. Specific Genetic Modifications Contained Within *E. coli* K12 strain E638

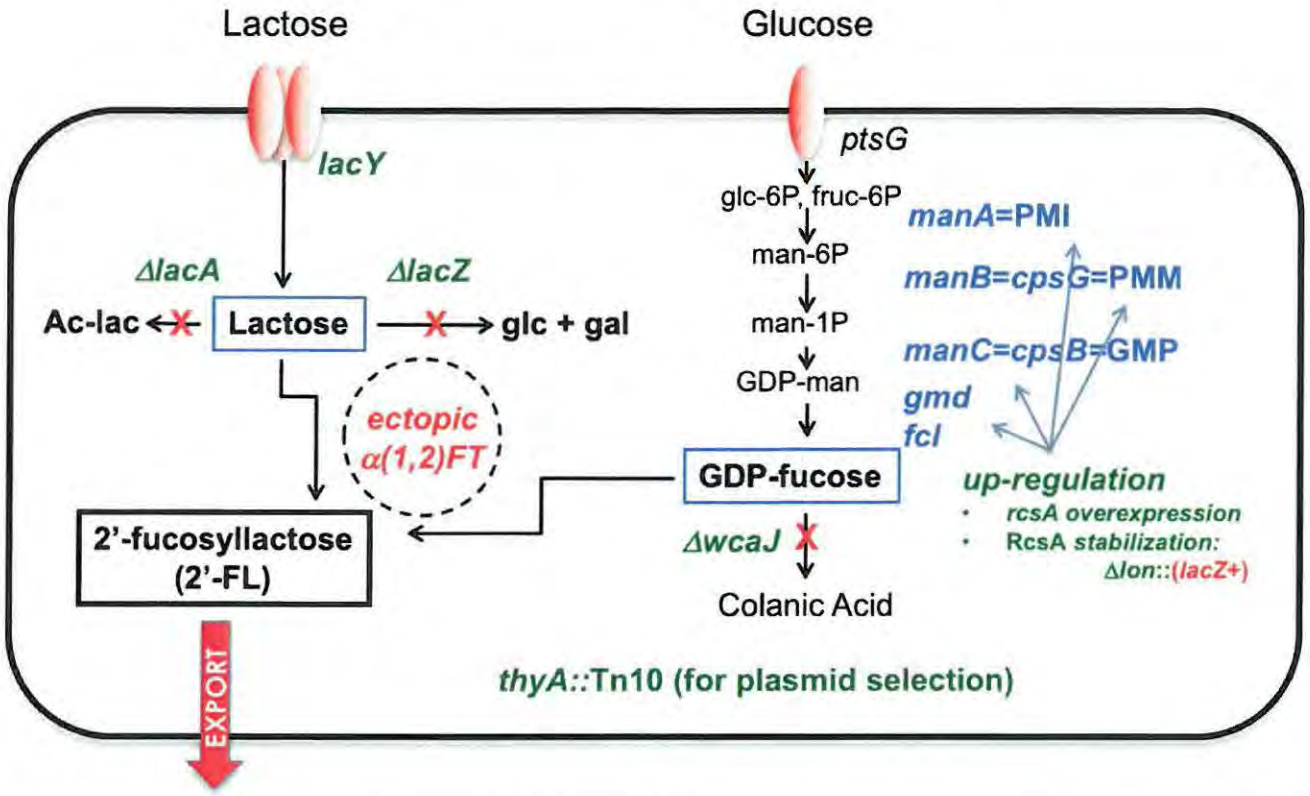
E. coli Gene	Gene Function	Modification Introduced	New Locus Designation	Purpose
lacA	Lactose acetylase	Complete deletion	*ΔlacA398*	Eliminates the production of acetyl-lactose in the cell.
lacI	*lac* repressor	Complete deletion	Δ(*lacI-lacZ*)*158*	Removes the endogenous *lacZ* gene encoding wild-type levels of β-galactosidase activity, enabling development of a cytoplasmic lactose pool. Positions a strong constitutive promoter upstream of *lacY*, the lactose permease gene.
lacZ	β-galactosidase	Complete deletion		
lacY	Lactose permease	Gene translocation	P*laclq-lacY*	Positions the *lacY* gene downstream from a strong constitutive promoter.
lon	ATP-dependent protease	Complete deletion and replacement with a gene cassette containing a promoterless *lacZ* CDS and a kanamycin resistance marker	*Δlon*::(*lacZ*+, KAN)	Deletes Lon protease function and also re-introduces a low but useful level of β-galactosidase activity into the cell.
wcaJ	UDP-glucose:undecaprenyl-phosphate glucose-1-phosphate transferase	Complete deletion	Δ*wcaJ134*	Eliminates the production of colanic acid, and enables accumulation of a cytoplasmic GDP-fucose pool.

***E. coli* Gene**	**Gene Function**	**Modification Introduced**	**New Locus Designation**	**Purpose**
thyA	Thymidylate synthase	Insertional inactivation	*thyA748*::Tn10	Introduces an auxotrophy to be used later for plasmid maintenance. **Note**: (Tn10 carries a tetracycline resistance marker).
ampC	*E. coli* K12 endogenous β-lactamase	Insertional inactivation	*ampC*::(P_{trp} λcI+)	Introduces a wild-type lambda repressor gene under the control of a tryptophan promoter.

Techniques of classical generalized phage transduction utilizing P1vir (Thomason et al., 2007) and λ Red recombineering (Datsenko and Wanner, 2000) were used in the construction of strain E638. Only three DNA segments in strain E638 are not formally derived from ancestral wild-type *E. coli* K12; the *cI*+ repressor gene (from *E. coli* bacteriophage lambda), the transposon Tn10 (bearing a tetracycline resistance gene), and the kanamycin resistance gene. Each of these three non-*E. coli* K12 DNA segments are nevertheless found commonly in laboratory and wild-type isolates of non-pathogenic *E. coli.* Note that the *thyA* auxotrophy of E638 prevents endogenous DNA synthesis by the strain, and that this lesion is lethal unless the defect is complemented by a plasmid-borne *thyA* gene, or unless the growth medium is supplemented with thymidine. Further details regarding the strain construction strategy of Glycosyn's *E. coli* K12 production host may be found in US patents 9,029,136 and 9,453,230 (Heidtman et al., 2015; Merighi et al., 2016).

The genetic changes that enable the production of 2'-FL in *E. coli* K12 are detailed in the schematic in Figure 2.

Figure 2. 2'-Fucosyllactose Production in Engineered *E. coli* K12



The presence of two cytoplasmic precursor pools is required for 2'-fucosyllactose production:

1. **The Lactose Pool**: *E. coli* K12 naturally possesses the ability to transport lactose from its environment and to use the sugar as a carbon source for growth. Several modifications of the lactose (*lac*) utilization operon were performed in engineered strain E638 to prevent this lactose catabolism and to promote development of a robust cytoplasmic lactose pool. First, lactose degradation through the action of endogenous β-galactosidase was prevented by deletion of the endogenous *lacZ* gene. Second, lactose transport from the culture medium into the cell was enhanced by placing the endogenous lactose permease gene (*lacY*) under a strong constitutive promoter. Finally, the production of undesirable acetyl-lactose was eliminated by deleting the endogenous *lacA* gene (acetyl-lactose presents a downstream purification challenge); and

2. **The GDP-Fucose Pool:** Wild-type *E. coli* K12 produces GDP-fucose for the sole purpose of making colanic acid, a fucose-containing polysaccharide containing a repeat unit with D-glucose, L-fucose, D-galactose, and D-glucuronate that is used in extracellular capsule formation. Elimination of the ability to make colanic acid has no deleterious effects on *E. coli* K12 growth in the bioreactor. In engineered strain E638, colanic acid production is eliminated downstream of GDP-fucose by removal of the *wcaJ* gene, and this single mutation leads to an accumulation of cytoplasmic GDP-fucose. The concentration of this GDP-fucose pool is then enhanced by inducing the earlier steps of the colanic acid synthesis pathway. This is achieved by increasing the level of the pathway's positive

transcriptional activator protein, RcsA, by: a) including the *rcsA* gene on a multicopy expression plasmid introduced into E638 (see below, the description of plasmid pG217); and b) eliminating the gene for Lon, the major protease responsible for RcsA turnover in the cell. As part of the *lon* deletion, a weak *lacZ* (β-galactosidase) allele was purposefully reintroduced into strain E638. The low level of β-galactosidase produced by this weak *lacZ* allele aids in downstream 2'-FL purification by removing residual lactose precursor at the end of fermentations, while not adversely impacting overall 2'-FL fermentation titers.

Lactose and GDP-fucose are efficiently and specifically converted into 2'-fucosyllactose by the action of the enzyme FutN, an α1,2 fucosyltransferase first identified at Glycosyn in the genome of the gut commensal organism, *Bacteroides vulgatus* ATCC 8482 (Heidtman et al., 2015).

Production of FutN enzyme is achieved in E638 by first transforming the strain with plasmid pG217 to generate a new strain, designated as "E997." pG217 carries the *futN* gene in an expression construct downstream of the strong inducible transcriptional promoter, pL. This promoter maintains an "off" status in minimal medium due to the presence of the wild-type cI repressor protein, expressed in the host strain under these conditions from a single copy chromosomal gene under the control of a trp promoter. To induce 2'-FL synthesis, tryptophan is added to the growth medium, which turns off production of cI repressor and slowly changes the pL promoter status to "on." As a consequence, *futN* is expressed and, if lactose is present, 2'-FL synthesis begins.

A map of plasmid pG217 is provided in Figure 3, and a detailed listing of the genes and elements carried on this plasmid is provided in Table 2.

Figure 3. Map of the 2'-Fucosyllactose Production Plasmid, pG217

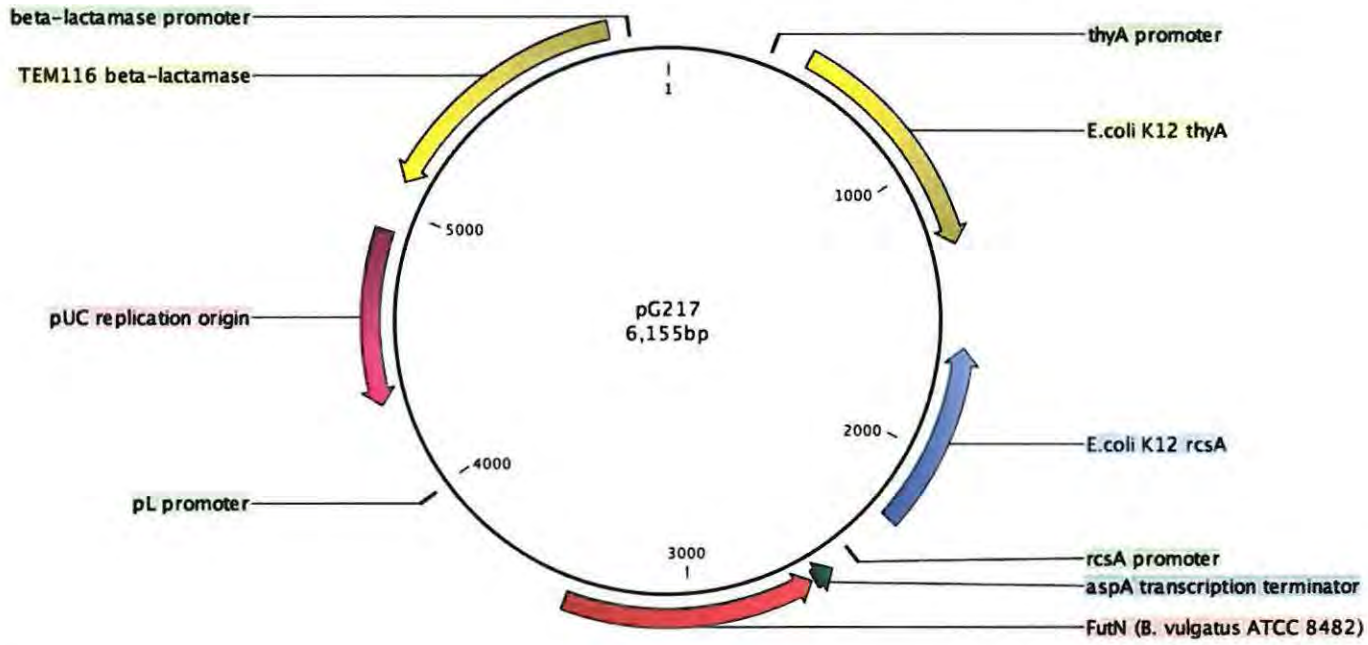


Table 2. Details of the Genes and Elements Carried on Plasmid pG217[a]

Nucleotide position	Gene or element name	Source	Purpose
1-242 bp	Vector DNA, no genes or elements	pUC19[b]	No function
243-1365 bp	Thymidylate synthase gene (*thyA*), includes the gene promoter	*E. coli* K12	Complements the *thyA* auxotrophy of E638, ensuring pG217 plasmid maintenance in minimal media
1366-1529 bp	Vector DNA, no genes or elements	pUC19	No function
1530-2495 bp	Colanic acid operon transcription activator protein gene (*rcsA*), includes the gene promoter	*E. coli* K12	Up-regulates genes responsible for production of GDP-fucose[c]
2496-2574 bp	*aspA* transcription terminator	*E. coli* K12	Terminates transcription from the upstream pL promoter
2574-3446 bp	Synthetic *futN* gene encoding an α1,2 fucosyltransferase (YP_001300461)	*Bacteroides vulgatus* ATCC 8482	Synthesis of 2'-fucosyllactose from lactose and GDP-fucose
3447-4280 bp	DNA segment carrying the bacteriophage lambda strong leftwards promoter, pL	*E. coli* K12 phage lambda (λ)	Controlled expression of the *futN* gene
4281-4335 bp	Vector DNA, no genes or elements	pUC19	No function
4336-4924 bp	pUC origin of replication	pUC19	Maintains pG217 plasmid copy number of ~25 copies per cell
4925-6155 bp	TEM116 β-lactamase gene, includes the gene promoter	pUC19	Encodes ampicillin resistance[d]

[a] pG217 is 6,155 base pairs (bp) long.

[b] Yanisch-Perron et al. (1985)

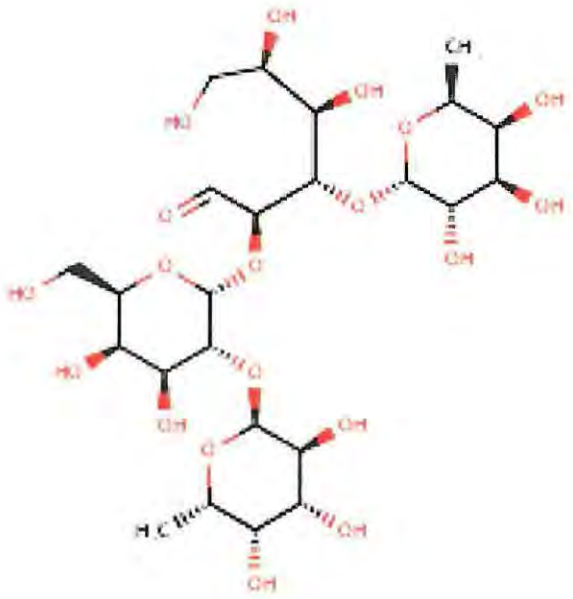
[c] The presence of multiple copies of the *rcsA* gene on pG217 boosts the cellular production of GDP-fucose.

[d] Inclusion of the TEM116 β-lactamase gene in pG217 was for operational convenience during the original construction of the plasmid. The gene is not utilized at any stage in the production of 2'-fucosyllactose, and at no stage is ampicillin or any other antibiotic present in the process (i.e. during the laying down of master and working cell banks, in the growth of seed cultures for production, or at any time in bioreactor fermentations).

3. Chemical Identity of Fermentation-Produced 2'-FL

The chemical identity of 2'-FL produced by Glycosyn and FrieslandCampina's *E. coli* strain E997 was confirmed by nuclear magnetic resonance (NMR) spectroscopy performed at the University of Georgia Complex Carbohydrate Research Center (CCRC, USA), at the University of Groningen (The Netherlands), and by Spectral Service (Cologne, Germany). Low levels (<2% total) of two other human milk sugars, 3-fucosyllactose (3-FL) and lactodifucotetraose (LDFT, Figure 4) were detected in samples of 2'-FL produced by E997. NMR reports from these analyses are provided in Appendix 1.

Figure 4. Structural Formula of Lactodifucotetraose[a]



[a] From ChemIDplus (2016b)

There are no known toxicants derived in the production of the 2'-FL material, and no constituents of the ingredient are known to effect safety.

B. Manufacturing Processes

Glycosyn and FrieslandCampina's 2'-FL is produced through the enzymatic transfer of fucose to lactose in an α-1,2-linkage. The reaction is catalyzed by fucosyltransferase present in an engineered host strain of *E. coli* K12 bacteria, discussed in Part 2.2. The 2'-FL production process consists of two stages: fermentation and purification. All raw materials used in the manufacturing process are suitable food-grade or better materials, and are used in accordance with applicable US Federal Regulations, as detailed in Table 3. Certificates of Analysis and/or specifications for the raw materials are provided in Appendix 2. Furthermore, all processing aids are suitable for use in food manufacturing, and are compliant with applicable US Federal Regulations, as defined in 21 CFR 173.25, 21 CFR 177.2440, and 21 CFR 173.340.

Table 3. Raw Materials and Processing Aids used to Manufacture Glycosyn/FrieslandCampina's 2'-FL

Name	CAS #	Function	Grade	Appendix Location
Raw Materials: Fermentation				
Glucose monohydrate	77938-63-7	Energy and carbon, source, precursor 2'-FL	FCC[1]	Appendix 2.1
α-Lactose monohydrate	5989-81-1	Precursor 2'-FL	Codex Alimentarius	Appendix 2.2
Phosphoric acid	7664-38-2	Fermentation media ingredient	FCC	Appendix 2.3
Ammonium hydroxide	1336-21-6	Fermentation media ingredient	FCC	Appendix 2.4
di-Ammonium hydrogen phosphate	7783-28-0	Fermentation media ingredient	FCC	Appendix 2.5
Monopotassium phosphate	7778-77-0	Fermentation media ingredient	Ph. Eur.[2] & USP-NF[3]	Appendix 2.6
L- Tryptophan	73-22-3	Inducer 2'-FL production	FCC	Appendix 2.7
Magnesium sulfate heptahydrate	10034-99-8	Fermentation media ingredient	Ph. Eur. & USP-NF	Appendix 2.8
Citric acid	77-92-9	Fermentation media ingredient	FCC	Appendix 2.9
Iron (II) sulfate heptahydrate	7782-63-0	Fermentation media ingredient	USP-NF	Appendix 2.10
Manganese chloride tetrahydrate	13446-34-9	Fermentation media ingredient	USP	Appendix 2.11
Cobalt (II) chloride hexahydrate	7791-13-1	Ingredient fermentation	Ph. Eur.	Appendix 2.12
Cupric sulfate pentahydrate	7758-99-8	Fermentation media ingredient	FCC	Appendix 2.13
Boric acid	10043-35-3	Fermentation media ingredient	USP-NF	Appendix 2.14
Zinc sulfate heptahydrate	7446-20-0	Fermentation media ingredient	FCC	Appendix 2.15
Sodium hydroxide	1310-73-2	pH adjustment fermentation	FCC	Appendix 2.16
Sodium molybdate dihydrate	10102-40-6	Fermentation media ingredient	Ph. Eur.	Appendix 2.17
Name	**CAS #**	**Function**	**Regulatory Compliance**	
Processing Aids				
Alkoxylated fatty acid esters on vegetable base	--	Antifoam-agent	Compliant with 21CFR173.340	
Cationic resin:	--	Purification aid	Compliant with 21CFR173.25	

Strong acid cation, styrene-DVB gel matrix, functional group sulfonic acid			
Anionic resin: Weak basic anion, crosslinked acrylic gel structure, functional group tertiary amines	--	Purification aid	Compliant with 21 CFR 177.2910
Absorbent resin: Absorbent, styrene divinylbenzene copolymer, functional group sulfonic acid	--	Purification aid	Compliant with 21 CFR 173.25
Microfiltration membrane	--	Filtration aid	Compliant with 21 CFR 177.2910
Ultrafiltration membrane	--	Filtration aid	Compliant with 21 CFR 177.2910
Nanofiltration membrane	--	Filtration aid	Compliant with 21 CFR subsections 177.1655, 177.2220, 177.1520, 175.105, 175.300, 177.2420, & 177.2800
RO membrane	--	Filtration aid	Compliant with 21 CFR
Citric acid	77-92-9	pH adjustment concentrate	FCC; See Appendix 2.9
Activated carbon Granular or powder	7440-44-0	Purification aid	FCC: See Appendix 2.18
MF membrane	--	Sterile filtration	Compliant with 21 CFR 177.2440 & 21 CFR 177.2600

[1] Food Chemicals Codex

[2] European Pharmacopoeia

[3] United States Pharmacopeia and The National Formulary

1. Fermentation

The fermentation for the production of Glycosyn and FrieslandCampina's 2'-FL is performed in a well-defined minimal medium and under sterile conditions. The energy and carbon source for the microorganism, and a precursor for 2'-FL, is glucose. The other precursor for 2'-FL is the disaccharide α-lactose, which is comprised of the monomers galactose (Gal) and glucose (Glc). The other components in the medium include: citric acid, ammonium, sodium, magnesium, phosphate, potassium, sulphate, antifoaming agents, and trace elements. No yeast extract, hydrolysates, chelating agents, or antibiotics are added. Demineralized water is used for highest purity.

Temperature, pH, and dissolved oxygen are controlled within defined limits during the fermentation process.

A "Master Cell Bank" (MCB) culture derived from a single isolated colony of the strain of interest (E997) is picked from an agar plate (Ferm4a medium +1.5% agar + 40 μg per mL X-Gal) previously grown at 30°C for 2 days. The colony is placed into 50 mL of Ferm4a liquid medium

contained within a 250-mL sterile disposable polyethylene terephthalate glycol-modified (PETG) baffled Nalgene Erlenmeyer flask with a 0.22 micron vented closure ("MCB flask"). The MCB flask is incubated at 30°C for 15-20 hours with agitation (250 rpm), until the OD_{600} of the culture reaches between 0.5 to 1.5. A MCB is produced by adding 70 μL of dimethylsulfoxide (DMSO) to several sterile 1.5-mL cryogenic polypropylene tubes and adding 1-mL aliquots of the MCB flask culture. The tubes are mixed by brief vortexing and then maintained at -80°C.

To prepare a "Working Cell Bank" (WCB) culture, a single MCB vial is thawed at room temperature for ~10 minutes, and 100 μL of the thawed culture is used to inoculate 200 mL of Ferm4a liquid medium contained in a 1,000-mL sterile disposable PETG baffled Nalgene Erlenmeyer flask with a 0.22 micron vented closure ("WCB flask"). The WCB flask is incubated at 30°C for 15-20 hours with agitation (250 rpm), until the OD_{600} of the culture reaches between 0.5 to 1.5. The WCB culture is then prepared as follows: 70 μL of DMSO is placed into several sterile 1.5-mL cryogenic polypropylene tubes, and 1-mL aliquots of the WCB flask culture are added to each tube. The tubes are mixed by brief vortexing and then placed in a -80°C freezer for storage. Tubes are maintained at -80°C until one is needed to seed a bioreactor run.

Each fermentation starts with a new and fresh WCB vial containing the E997 production strain in a frozen form. After thawing, an accurate volume of the culture is transferred from the vial to several shake flasks that are grown in parallel to form the starter culture for a seed fermenter. The seed fermenter is transferred to the main fermenter when a predefined culture density is reached. The media in these stages are also free of yeast extract, hydrolysates, chelating agents, and antibiotics, and the conditions are the same.

Induction of 2'-FL production, after an initial phase of batch growth of the culture on glucose to build biomass, is accomplished by the addition of the amino acid L-tryptophan. The culture is then grown in fed batch under a regime where constant feeds of glucose and lactose are provided, and during this phase 2'-FL is excreted into the medium. The fermentation stops when the precursors have been consumed. Subsequently, the fermentation broth is sent to stage 2: purification.

2. Purification

After fermentation (production phase), the intact microbiological cells are separated from the liquid by microfiltration at 40-50°C. The cells do not have to be lysed because the product has been excreted into the medium. Diafiltration is applied to increase the recovery from the broth. The biomass residue is inactivated with a caustic treatment. After microfiltration, a heat treatment is applied to ensure improved processing in the rest of the purification process. The heat treatment is followed by ultrafiltration to remove protein and some peptide particles. Diafiltration is also applied to increase the yield.

Next, column chromatography with a combination of cationic, anionic, and absorbent resins is used to remove cations, anions, organic impurities, and color. The concentration of 2'-FL at the outlet of the chromatography step is very low (<4%); therefore, a membrane concentration step is applied

(nanofiltration or reverse osmosis) to concentrate the product up to 20-30% 2'-FL on a dry basis. The pH is adjusted with citric acid when required. The concentrate is pasteurized at a minimum of 20 seconds at a minimum 72°C, and spray dried in a spray dryer to obtain high purity 2'-FL powder.

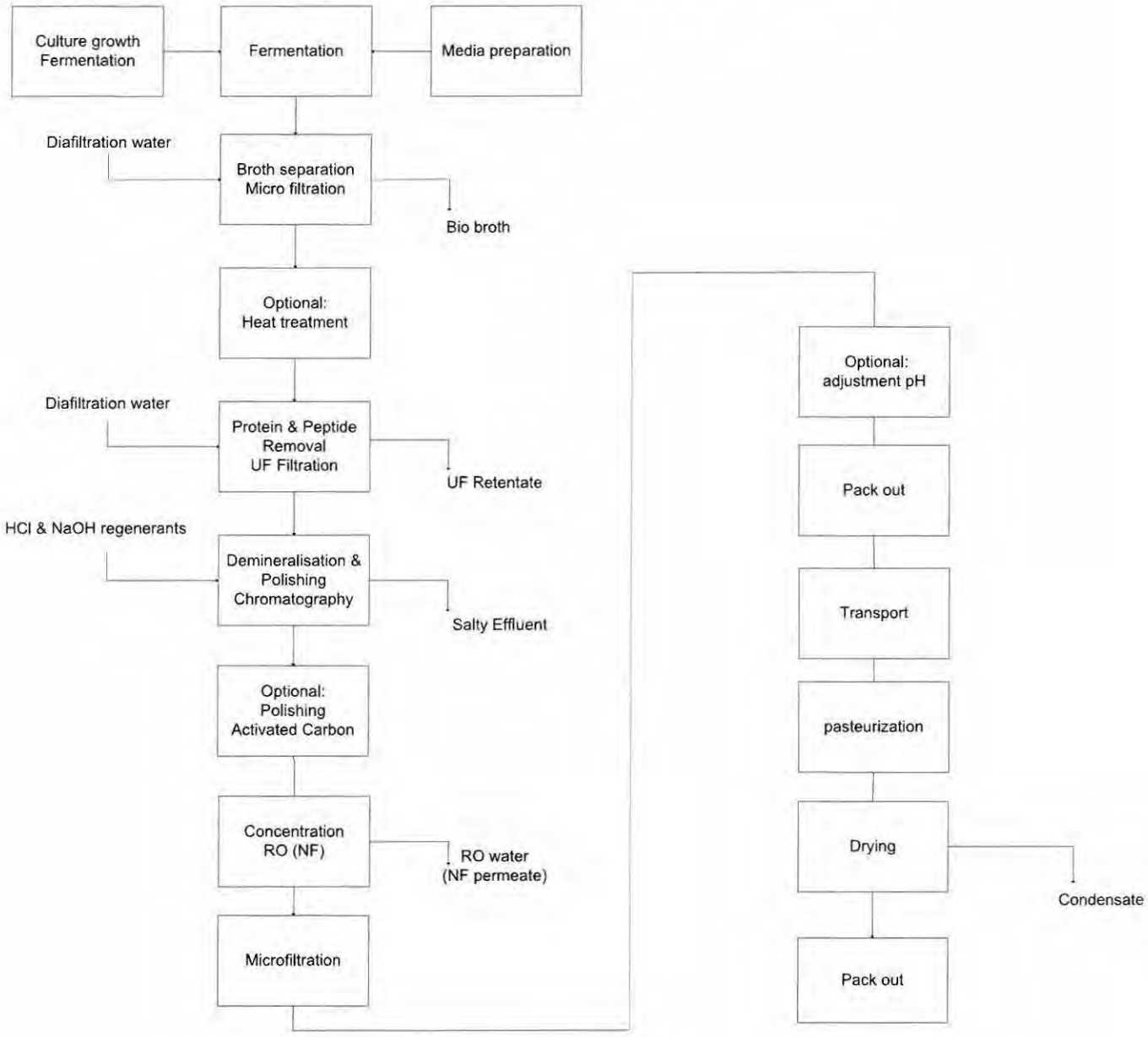
An overview of the production process and a manufacturing flow chart are provided in Table 4 and Figure 5, respectively.

Table 4. Overview of Glycosyn/FrieslandCampina's Production Process for 2'-FL

#	Step	Function
F1	Flask inoculation	Grow strain from cell bank
F2	Seed fermentation	Produce enough biomass to inoculate main fermenter
F3	Main fermentation	Biomass growth for 2'-FL production
P1	Microfiltration	Separation of cells and supernatant with product (2'-FL)
P2	Heat treatment	Optional: Improved processing behavior: no impact on product
P3	Ultrafiltration	Removal of proteins and other larger cell debris
P4	Chromatography	Removal of ions, organic impurities like protein and DNA fragments, and color by absorption
P5	Activated carbon	Optional: Removal of minor impurities
P6	Nanofiltration or reverse osmosis	Concentration of the product through the removal of water
P7	Microfiltration	Removal of potential microbiological contamination
P8	pH adjustment	Optional
P9	Sampling and packaging	Intermediate product is packaged
P10	Quality control	
P11	Transport	Transport to drying location
P12	Pasteurization	Removal of potential microbiological contamination
P13	Drying	Convert concentrate with a (spray) drying process into powder
P14	Sampling and packaging	Intermediate product is packaged
P15	Quality control	
P16	Release	

F represents fermentation steps; P represents purifications steps.

Figure 5. Flowchart of Manufacturing Process



C. Product Specifications

The European Union (EU) recently published specifications for 2'-*O*-Fucosyllactose[2] (EU, 2016), as presented by Glycom and provided in Table 5 below.

Table 5. EU Specifications for 2'-*O*-Fucosyllactose

Physical and Chemical Parameters	EU Specifications
Appearance, Form, and Color	White to off-white powder
Assay	Not less than 95%
D-Lactose	Not more than 1.0 w/w%
L-Fucose	Not more than 1.0 w/w%
Difucosyl-d-lactose isomers	Not more than 1.0 w/w%
2'-Fucosyl-d-lactulose	Not more than 0.6 w/w%
pH (20 °C, 5% solution)	3.2-7.0
Water (%)	Not more than 9.0%
Ash, Sulfated	Not more than 0.2%
Acetic Acid	Not more than 0.3%
Residual solvents (methanol, 2-propanol, methyl acetate, acetone)	Not more than 50 mg/kg singly Not more than 200 mg/kg in combination
Residual Proteins	Not more than 0.01%
Palladium	Not more than 0.1 mg/kg
Nickel	Not more than 3.0 mg/kg
Aerobic Mesophilic Bacteria Total Count	Not more than 500 CFU/g
Yeasts	Not more than 10 CFU/g
Moulds	Not more than 10 CFU/g
Residual Endotoxins	Not more than 10 EU/mg

Other than the recent EU specifications detailed in Table 5, no other established regulatory specifications for 2'-fucosyllactose have been identified. However, multiple GRAS notifications

[2] 2-*O*-Fucosyllactose is a synonym for 2'-fucosyllactose.

(GRNs) have established product specifications for their 2'-FL products (Glycom, 2014; Jennewein, 2015), and a "no questions" letter was issued by FDA for each notification.

Glycosyn and FrieslandCampina have likewise adopted similar product specifications for their purified 2'-fucosyllactose preparation. It should be noted that no specifications for residual solvents or catalysts have been established, as published by the EU in Table 5, since Glycosyn and FrieslandCampina's production process does not require the use of solvents or catalysts.

Specifications for 2'-FL produced by Glycosyn and FrieslandCampina are compared in Table 6 to those specifications provided by Glycom (2014) and Jennewein (2015). Results of analyses performed by FrieslandCampina demonstrate that the five production batches of 2'-FL meet the designated specifications, as shown in Table 7.

Details of the analytical methodology employed to determine 2'-fucosyllactose are provided in Appendix 3, the chromatograms for representative 2'-FL preparations are provided in Appendix 4, and certificates of analysis for five representative lots of 2'-FL are provided in Appendix 5. A report detailing the quantitative polymerase chain reaction (qPCR) method to evaluate 2'-FL for the absence of residual genetic material from the *E. coli* production strain is provided in Appendix 6. The collection of these reports demonstrates that the substance is well characterized and meets the established purity criteria.

Table 6. Specifications for Purified 2'-Fucosyllactose Preparations

Physical and Chemical Parameters	Glycom[a] 2'-FL Specifications (GRN 546)	Glycom[b] 2'-FL Specifications (GRN 650)	Jennewein[c] 2'-Fucosyllactose Specifications	EU[d] 2'-*O*-Fucosyllactose Specifications	Glycosyn/ FrieslandCampina Specifications
Appearance Form	Powder	Powder	NS	Powder	Homogenous powder
Appearance Color	White to off white	White to off white	NS	White to off white	White
Assay	Min. 95.0% (HPLC, water free)	Min. 94.0% (HPLC, water free)	≥ 90% (HPAEC-PAD area)	Not less than 95%	Min. 90% (HPAEC)
pH	3.0-7.5 (20 °C, 5% solution)	3.2-5.0 (20 °C, 5% solution)	NS	3.2-7.0 (20 °C, 5% solution)	3.0-7.5 (10% solution)
Water	Max. 9.0%	Max. 5.0%	≤ 9.0%	Not more than 9.0%	Max. 5%
Ash	Max. 0.2% (Sulphated)	Max. 1.5%	≤ 0.5%	Not more than 0.2% (sulfated)	Max. 0.2% (sulfated)
Acetic acid (as free acid and/or sodium acetate)	Max. 0.3%	Max. 1.0%	NS	Not more than 0.3%	NS
Residual Solvents[e]	Max. 50 mg/kg singly Max. 200 mg/kg in combination	NS	NS	Not more than 50 mg/kg singly Not more than 200 mg/kg in combination	NS
Residual Proteins	0.1%	0.01%	≤ 100 µg/g	Not more than 0.01%	Max. 0.01%
Palladium	NS	NS	NS	Not more than 0.1 mg/kg	NS
Nickel	NS	NS	NS	Not more than 3.0 mg/kg	NS
Aluminum	NS	NS	NS	NS	Max. 4.8 mg/kg
Lead	Max. 0.8 mg/kg	Max 0.1 mg/kg	≤ 0.02 mg/kg	NS	Max. 0.05 mg/kg
Arsenic	NS	NS	≤ 0.2 mg/kg	NS	Max. 0.1 mg/kg

Physical and Chemical Parameters	Glycom[a] 2'-FL Specifications (GRN 546)	Glycom[b] 2'-FL Specifications (GRN 650)	Jennewein[c] 2'-Fucosyllactose Specifications	EU[d] 2'-*O*-Fucosyllactose Specifications	Glycosyn/ FrieslandCampina Specifications
Cadmium	NS	NS	≤ 0.1 mg/kg	NS	Max. 0.01 mg/kg
Mercury	NS	NS	≤ 0.5 mg/kg	NS	Max. 0.05 mg/kg
Lactose	NS	Max. 3.0 w/w%	≤ 5%	Not more than 1.0 w/w%	Max. 3%
3-Fucosyllactose	NS	NS	≤ 5%	NS	NS
Difucosyllactose	NS	Max. 1.0 w/w%	≤ 5%	NS	NS
Fucosyl-Galactose	NS	NS	≤ 3%	NS	NS
2'-Fucosyl-D-lactulose	NS	Max. 1.0 w/w%	NS	NS	NS
Allo-lactose	NS	NS	NS	NS	Max. 2%
Glucose	NS	NS	≤ 3%	NS	Max. 2%
Galactose	NS	NS	≤ 3%	NS	Max. 2%
Fucose	NS	Max. 1.0 w/w%	≤ 3%	Not more than 1.0 w/w%	Max. 2%
Nitrite	NS	NS	NS	NS	Max. 1 mg/kg
Nitrate	NS	NS	NS	NS	Max. 50 mg/kg
Scorched particles	NS	NS	NS	NS	Max. disc A
Aerobic mesophilic total count	Max. 500 CFU/g	Max. 500 CFU/g	≤ 10,000 cfu/g[f]	Not more than 500 CFU/g	Max. 3,000 cfu/g
Yeast	Max. 10 CFU/g	Max. 10 CFU/g	≤ 100 cfu/g	Not more than 10 CFU/g	Max. 10 cfu/g
Mold	Max. 10 CFU/g	Max. 10 CFU/g		Not more than 10 CFU/g	Max. 10 cfu/g

Physical and Chemical Parameters	Glycom[a] 2'-FL Specifications (GRN 546)	Glycom[b] 2'-FL Specifications (GRN 650)	Jennewein[c] 2'-Fucosyllactose Specifications	EU[d] 2'-*O*-Fucosyllactose Specifications	Glycosyn/ FrieslandCampina Specifications
Salmonella	Absent in 25 g	Absent in 25 g	Absent in 100 g	NS	Absent in 25 g
Enterobacteriaceae	Absent in 10 g	Absent in 10 g	Absent in 11 g (with Coliform)	NS	Absent in 10 g
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Absent in 10 g	Absent in 100 g	NS	Absent in 25 g
Listeria monocytogenes	Absent in 25 g	Absent in 25 g	NS	NS	NS
Bacillus cereus	Max. 50 CFU/g	Max. 50 CFU/g	NS	NS	Max. 100 cfu/g (presumptive)
E. coli	NS	NS	NS	NS	Absent in 10 g
Staphylococcus aureus	NS	NS	NS	NS	Absent in 1 g
Sulphite reducing clostridia spores	NS	NS	NS	NS	Max. 30 cfu/g
Clostridium perfringens	NS	NS	NS	NS	Absent in 1 g
Residual Endotoxins	Max. 50 EU/mg	NS	≤ 300 EU/g	Not more than 10 EU/mg	Max. 10 EU/mg
Aflatoxin M_1	NS	NS	≤ 0.025 µg/kg	NS	Max. 0.2 µg/kg
GMO detect ion	NS	NS	Negative	NS	Negative

[a] Glycom (2014), 2'-FL produced by chemical synthesis.

[b] Glycom (2016), 2'-FL produced by fermentation.

[c] Jennewein (2015), 2'-FL produced by fermentation.

[d] European Union specifications for 2'-*O*-Fucosyllactose (EU, 2016).

[e] Residual solvents, specifically: methanol, 2-propanol, methyl acetate, and/or acetone.

[f] Standard plate count

NS = Not specified; RT = Retention Time; HPAEC-PAD = High-performance anion-exchange chromatography with pulsed amperometric detection

Table 7. Analytical Results for 5 Nonconsecutive Lots of 2'-FL

Physical and Chemical Parameters	Glycosyn/ FrieslandCampina Specifications	Results of Batch Numbers				
		PMRS10	PMRS11	CMRS03	CMRS06	CMRS07
Appearance Form	Homogenous powder	Pass	Pass	Pass	Pass	Pass
Appearance Color	White	Pass	Pass	Pass	Pass	Pass
Assay	Min. 90% (HPAEC)	94.2%	93.2%	93.3%	92.7%	93.8%
pH	3.0-7.5 (10% solution)	4.11	4.0	3.64	4.03	5.07
Water	Max. 5%	3.83%	3.33%	4.19%	3.59%	3.6%
Ash	Max. 0.2% (sulfated)	0.02%	0.03%	0.02%	0.1%	0.08%
Residual Proteins	Max. 0.01%	< 0.01%	< 0.01%	< 0.01%	< 0.01%	< 0.01%
Aluminum	Max. 4.8 mg/kg	< 0.2 mg/kg	< 0.2 mg/kg	< 0.2 mg/kg	< 0.2 mg/kg	< 0.4 mg/kg
Lead	Max. 0.05 mg/kg	< 0.02 mg/kg	< 0.02 mg/kg	< 0.02 mg/kg	< 0.02 mg/kg	< 0.02 mg/kg
Arsenic	Max. 0.1 mg/kg	< 0.010 mg/kg	< 0.01 mg/kg	< 0.010 mg/kg	< 0.01 mg/kg	< 0.020 mg/kg
Cadmium	Max. 0.01 mg/kg	< 0.005 mg/kg	< 0.005 mg/kg	< 0.005 mg/kg	< 0.005 mg/kg	< 0.010 mg/kg
Mercury	Max. 0.05 mg/kg	< 0.003 mg/kg	< 0.003 mg/kg	< 0.003 mg/kg	< 0.006 mg/kg	< 0.011 mg/kg
Lactose	Max. 3%	0.6%	0.5%	1.1%	0.8%	0.5%
Allo-lactose	Max. 2%	0.5%	0.6%	0.6%	0.7%	1.0%
Glucose	Max. 2%	0.1%	0.1%	0.1%	0.1%	< 0.1%
Galactose	Max. 2%	< 0.1%	< 0.1%	< 0.1%	< 0.1%	< 0.1%
Fucose	Max. 2%	0.2%	0.2%	0.4%	0.1%	0.2%

Physical and Chemical Parameters	Glycosyn/ FrieslandCampina Specifications	Results of Batch Numbers				
		PMRS10	PMRS11	CMRS03	CMRS06	CMRS07
Nitrite	Max. 1 mg/kg	< 0.1 mg/kg	< 0.1 mg/kg	< 0.1 mg/kg	< 0.1 mg/kg	< 0.1 mg/kg
Nitrate	Max. 50 mg/kg	0.7 mg/kg	1.0 mg/kg	0.3 mg/kg	1.6 mg/kg	0.9 mg/kg
Scorched particles	Max. disc A	A	A	A	A	A
Aerobic mesophilic total count	Max. 3,000 cfu/g	300 cfu/g	< 1,000 cfu/g	< 1,000 cfu/g	< 1,000 cfu/g	< 1,000 cfu/g
Yeast	Max. 10 cfu/g	< 1 cfu/g	< 1 cfu/g	< 1 cfu/g	< 1 cfu/g	< 1 cfu/g
Mold	Max. 10 cfu/g	< 1 cfu/g	< 1 cfu/g	< 1 cfu/g	< 1 cfu/g	< 1 cfu/g
Salmonella	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g
Enterobacteriaceae	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g
Cronobacter (Enterobacter) sakazakii	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g
Bacillus cereus	Max. 100 cfu/g (presumptive)	10 cfu/g	< 1 cfu/g	30 cfu/g	< 1 cfu/g	20 cfu/g
E. coli	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g
Staphylococcus aureus	Absent in 1 g	Absent in 1 g	Absent in 1 g	Absent in 1 g	Absent in 1 g	Absent in 1 g
Sulphite reducing clostridia spores	Max. 30 cfu/g	1 cfu/g	< 1 cfu/g	< 1 cfu/g	< 1 cfu/g	< 1 cfu/g
Clostridium perfringens	Absent in 1 g	Absent in 1 g	Absent in 1 g	Absent in 1 g	Absent in 1 g	Absent in 1 g
Residual Endotoxins	Max. 10 EU/mg	0.88 EU/mg	0.54 EU/mg	0.21 EU/mg	< 0.1 EU/mg	< 0.1 EU/mg
Aflatoxin M_1	Max. 0.2 µg/kg	< 0.1 µg/kg	< 0.1 µg/kg	< 0.1 µg/kg	< 0.1 µg/kg	< 0.1 µg/kg
GMO detection	Negative	Negative	Negative	Negative	Negative	Negative

RT = Retention Time; HPAEC-PAD = High-performance anion-exchange chromatography with pulsed amperometric detection

D. Physical or Technical Effect

Human milk oligosaccharides (HMOs) are a structurally diverse group of unconjugated carbohydrates that are composed of the building blocks D-glucose, D-galactose, *N*-acetylglucosamine, L-fucose, and sialic acid. The basic structure contains a lactose at the reducing end of the HMO. Modification at the galactose or glucose moieties with fucose, sialic acid, or N-acetylglucosamine in different types of glycosidic linkages leads to the generation of an array of highly complex structures (Bode and Jantscher-Krenn, 2012). HMOs that are modified with sialyl or sulfate groups are considered acidic oligosaccharides, whereas unmodified or fucosylated HMOs are classified as neutral. The oligosaccharide fraction in human milk consists of 80-85% neutral and 15-20% acidic HMOs.

The proportion of acidic and neutral oligosaccharides, and the individual composition of their compounds, is one of the major differences between human milk and milk from domestic animals. Whereas human milk contains up to 85% neutral HMOs, only 9% of bovine milk oligosaccharides are neutral. In addition, only 3 of the 12 identified neutral oligosaccharide in bovine milk are fucosylated (Castanys-Munoz et al., 2013). 2'-FL is practically absent in bovine milk and was found in only very small amounts in the milk of goats and sheep (Albrecht et al., 2014).

HMOs play a pivotal role in the intestinal colonization by beneficial microbes, especially by *Bifidobacteria* and *Lactobacilli*, and protect the immature intestine against the colonization with pathogens (Newburg, 2013). They further seem to stimulate intestinal adaptation (Engfer et al., 2000) and may have systemic effects on the immune system of the infants.

The ingredient will be used in infant formula to provide equivalent levels of 2'-FL present in human milk. For toddlers and adults, 2'-FL is suggested to promote digestive health (Elison et al., 2016; Morrow et al., 2004)

E. Stability

1. Stability Data on 2'-FL

2'-Fucosyllactose has previously been reported to be stable under real-time and accelerated conditions for the bulk material, and under the intended conditions of use in certain applications including infant formula.

Glycom reported real-time shelf-stability (at 25°C and a relative humidity of 60%) of 36 months and stability under accelerated conditions (at 40°C and a relative humidity of 75%) for 6 months for its 2'-FL preparation. Furthermore, Glycom noted that 2'-FL was stable under the intended conditions of use in a variety of applications, including infant formula, yoghurt, citrus fruit drinks, and ready-to-drink chocolate-flavored milk when prepared and stored under recommended conditions (Glycom, 2014). More recently, Glycom indicated that its fermentation-produced 2'-FL has a calculated stability of 5 years when protected from light, and stored at room temperature under ambient

humidity. It was also noted that their fermentation-produced 2'-FL is stable under intended conditions of use in conventional foods and infant formula (Glycom, 2016).

Jennewein indicates that its 2'-FL preparation has a guaranteed shelf-life of at least 2 years (at 25°C and a relative humidity of 65%) and at least 6 months when stored at 40°C and a relative humidity of 75% (Jennewein, 2015).

2. Stability Data for Glycosyn and FrieslandCampina's 2'-FL Preparation

FrieslandCampina is currently conducting a 6-month accelerated storage and 36-month shelf-stability study on the subject 2'-FL preparation. For the accelerated stability study, samples of 2'-FL were stored at 40°C at a relative humidity of 75%, with pull dates of 0, 3, and 6 months. For the shelf-stability study, samples of 2'-FL were stored at 25°C at a relative humidity of 60%, with pull dates of 0, 3, and 6, 12, 24, and 36 months. The stability samples were then tested for assay, as well as a number of other chemical and microbiological parameters.

A summary of the available accelerated and shelf-stability results are presented in Table 8 and Table 9, respectively.

Table 8. Purified 2'-Fucosyllactose Accelerated Storage Stability Data

Parameter	Specifications	t= 0	t= 3 months	t = 6 months
Assay	Min. 90%	96.3%	95.0%	94.2%
Moisture	Max. 5%	3.3%	4.2%	3.7%
Ash	Max. 0.2%	0.11%	0.03%	0.02%
Lactose	Max. 3%	0.6%	1.1%	2.1%
Allo-lactose	Max. 2%	0.1%	1.2%	1.6%
Glucose	Max 2%	0.1%	0.1%	0.4%
Mesophilic aerobic cell count	Max. 3, 000 cfu/g	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g
Enterobacteriaceae	Absent in 10 g	Negative	Negative	Negative
Salmonella	Absent in 25 g	Negative	Negative	Negative
Cronobacter spp.	Absent in 25 g	Negative	Negative	Negative
Appearance	White homogenous powder	Pass	Pass	Pass

Table 9. Purified 2'-Fucosyllactose Shelf-Storage Stability Data

Parameter	Specifications	t= 0	t= 3 months	t = 6 months	t=12 months	t=24 months	t=36 months
Assay	Min. 90%	96.3%	98.2%	94.8%	NA	NA	NA
Moisture	Max. 5%	3.3%	3.7%	4.0%	NA	NA	NA
Ash	Max. 0.2%	0.11%	< 0.01%	0.01%	NA	NA	NA
Lactose	Max. 3%	0.6%	0.6%	1.3%	NA	NA	NA
Allo-lactose	Max. 2%	0.1%	1.1%	1.8%	NA	NA	NA
Glucose	Max 2%	0.1%	0.1%	0.3%	NA	NA	NA
Mesophilic aerobic cell count	Max. 3, 000 cfu/g	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g	NA	NA	NA
Enterobacteriaceae	Absent in 10 g	Negative	Negative	Negative	NA	NA	NA
Salmonella	Absent in 25 g	Negative	Negative	Negative	NA	NA	NA
Cronobacter spp.	Absent in 25 g	Negative	Negative	Negative	NA	NA	NA
Appearance	White homogenous powder	Pass	Pass	Pass	NA	NA	NA

NA = Not available, stability study is ongoing.

The stability data reported for 2'-FL in previous GRNs (Glycom, 2014; 2016; Jennewein, 2015), along with FrieslandCampina's stability testing results, support the position that Glycosyn and FrieslandCampina's purified 2'-fucosyllactose preparation is well-suited for the intended food uses.

PART 3. DIETARY EXPOSURE

A. Estimate of Dietary Exposure to the Substance

1. Intended Uses

The subject 2'-fucosyllactose preparation (> 90% 2'-FL) is intended to be used in a number of conventional foods and medical foods, as well as conventional infant formula for full term infants. No uses in pre-term infants are proposed at this time. Table 10 lists proposed conventional food categories, intended uses, and use levels for purified 2'-fucosyllactose. Food categories were selected from the U.S. National Center for Health Statistics' (NCHS) National Health and Nutrition Examination Surveys (NHANES) 2013-2014 survey, and were grouped according to 21 CFR 170.3. In certain cases, adjustment factors were prepared for composite foods and mixtures based on data contained in the Food and Nutrition Database for Dietary Studies (FNDDS). A detailed report on the estimated daily intake assessment is provided in Appendix 8.

Table 10. Proposed Conventional Food Categories and Intended Use

Proposed Food Category	Food Uses	Maximum 2'-FL Use Level (g/serving)	RACC[a] (g or mL)	Maximum 2'-FL Use Levels (g/100 g)
Beverages and Beverage Bases	Energy drinks	0.28	360	0.08
	Fitness water and thirst quenchers, sports and isotonic drinks	0.28	360	0.08
Breakfast Cereals	Ready-to-eat breakfast cereals for adults and children	1.2	15 (puffed) 40 (high-fiber) 60 (biscuit-types)	8.0 3.0 2.0
	Hot cereals for adults and children	1.2	40 (dry) ~250 prepared	0.48 (as consumed)
Dairy Product Analogs	Milk substitutes such as soy milk and imitation milks	0.28	240	0.12
Frozen Dairy Desserts and Mixes	Frozen desserts including ice creams* and frozen yogurts, frozen novelties	1.2	~70	1.7
Gelatins, Puddings, and Fillings	Dairy-based puddings, custards, and mousses	1.2	~70	1.7
	Fruit pie filling	1.2	85	1.41
	"Fruit prep" such as fruit filling in bars, cookies, yogurt, and cakes	1.2	~40	3.0
Grain Products and Pastas	Bars, including snack bars, meal-replacement bars, and breakfast bars	0.48	40	1.20
Jams and Jellies, Commercial	Jellies and jams, fruit preserves*, and fruit butters	1.2	~20	6.0
Milk, Whole and Skim	All *Acidophilus* or fortified milks, non-fat and low-fat milk fluids, including fluid milk and reconstituted milk powder*	0.28	240	0.12
Milk Products	Flavored milks, including chocolate milk, coffee drinks, cocoa, smoothies (dairy and fruit-based), other fruit and dairy combinations, yogurt drinks, and fermented milk drinks including kefir**	0.28	240	0.12
	Milk-based meal replacement beverages or diet beverages**	0.28	240	0.12
	Yogurt*, **	1.2	225	0.53
	Formula intended for pregnant women ("mum" formulas, -9 to 0 months)	1.2	200[b]	0.6
Processed Fruits and Fruit Juiced	Fruit drinks, including vitamin and mineral-fortified products	0.28	240	0.12
	Fruit juices*	0.28	240	0.12
Sweet sauces, Toppings, and Syrups	Syrups used to flavor milk beverages	0.28	40	0.70
Other Categories				

Non-Exempt Infant and Follow-On Formula	Infant formula (0 to 6 months), including ready-to-drink formula or formula prepared from powder	0.24	100[b]	0.24 (0.40 g/100 kcal)
	Follow-on formula (6-12 months), including ready-to-drink formula or formula prepared from powder	0.24	100[b]	0.24 (0.40 g/100 kcal)
	Infant meal replacement products such as PediaSure®	0.24	100[b]	0.24 (0.40 g/100 kcal)
Baby Foods	Growing-up (toddler) milks (12-36 months)	0.24	100[b]	0.24
	Ready-to-eat, ready-to-serve, hot cereals	1.2	15 (dry) 110 (ready-to-serve)	1.09 (as consumed)
	Yogurt and juice beverages identified as "baby" drinks	1.2	120	1.0
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts)	1.2	110	1.09
	Baby crackers, pretzels, cookies, and snack items	0.4	7	5.7
Medical Foods	Oral nutritional supplements and enteral tube feeding (11 years and older)	4.0	200[b]	2.0

[a] Reference Amounts Customarily Consumed per Eating Occasion (RACC), based on values established in 21 CFR 101.12. Note: when a range of values is reported for a proposed food use, particular foods within that food use may differ with respect to their RACC.

[b] No RACC value exists; therefore, approximate serving sizes are provided according to food manufacturer instructions.

* 2'-FL is intended for use in unstandardized products when standards of identity do not permit its addition.

** Includes ready-to-drink and powder forms.

a. Estimated Daily Intake from All Proposed Food Uses

Estimated daily intakes (EDIs) of 2'-FL were determined for the US population based on the proposed food uses presented in Table 10. The total estimated intakes of 2'-FL, determined as g per person per day and as mg per kg body weight (bw) per day, are summarized in Table 11 and Table 12, respectively. While both the *per capita* and consumer-only intakes are presented, the consumer-only EDIs represent the estimated exposures in the target population.

The mean and 90th percentile consumer-only intakes of 2'-FL in the total population (consumers of all ages) were determined to be 1.70 g per person per day and 3.54 g per person per day, respectively. Infants aged 6 to 11 months were determined to have the highest mean consumer-only intakes, at 2.28 g 2'-FL per person per day, while male teenagers were determined to have the highest 90th percentile intake, at 4.29 g 2'-FL per person per day. The lowest estimated mean and 90th percentile EDIs for 2'-FL were determined for females of childbearing age (aged 16 to 45 years), at 1.36 g per person per day and 2.87 g per person per day, respectively.

Consumer-only intakes of 2'-FL in the total population were also determined on a body weight basis, where the mean and 90th percentile estimates were determined to be 36 mg per kg bw per day and 80 mg per kg bw per day, respectively. Of all consumer-only population groups, infants aged 0 to 5 months were estimated to have the highest mean and 90th percentile, of 315 mg per kg

bw per day and 532 mg per kg bw per day, respectively. The lowest mean and 90th percentile EDIs for 2'-FL were determined for adult females and females of childbearing age, at 20 mg per kg bw per day and 43 mg per kg bw per day, respectively.

Table 11. Summary of the Estimated Daily Intake of 2'-FL from Proposed Uses by Population Group

Population Group	Age Group	*Per capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0-5 months	1.10	2.75	57.5	107	1.91	3.00
Infants	6-11 months	2.14	3.86	94.1	160	2.28	3.86
Toddlers	12-35 months	1.83	2.97	100.0	348	1.83	2.97
Children	3-11 years	1.96	3.53	99.7	1,277	1.97	3.53
Female Teenagers	12-19 years	1.47	2.95	94.7	544	1.55	2.95
Male Teenagers	12-19 years	1.85	4.16	92.5	526	2.00	4.29
Women of Child-Bearing Age	16-45 years	1.22	2.82	89.9	1,219	1.36	2.87
Female Adults	20 years and up	1.32	2.96	91.9	2,169	1.44	3.05
Male Adults	20 years and up	1.59	3.81	86.8	1,842	1.84	3.97
Elderly	65 years and up	1.76	3.74	92.8	939	1.90	3.91
Total Population	All ages	1.55	3.41	91.2	6,973	1.70	3.54

Table 12. Summary of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL from Proposed Uses by Population Group

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	0-5 months	181	477	57.5	107	315	532
Infants	6-11 months	244	441	94.1	160	259	447
Toddlers	12-35 months	148	243	100.0	346	148	243
Children	3-11 years	75	147	99.7	1,268	76	147
Female Teenagers	12-19 years	24	52	94.7	536	26	52

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
Male Teenagers	12-19 years	29	67	92.5	524	31	67
Women of Child-Bearing Age	16-45 years	18	42	89.9	1,209	20	43
Female Adults	20 years and up	19	42	91.9	2,156	20	43
Male Adults	20 years and up	19	46	86.7	1,833	22	48
Elderly	65 years and up	24	53	92.6	928	26	54
Total Population	All ages	32	76	91.1	6,930	36	80

b. Estimated Daily Intake for Non-Breastfed Infant and Toddler Subpopulations

To estimate the intake of 2'-FL from formula products, a subsequent assessment was performed in which the breastfed infant/toddler subpopulation was removed in order to predict the 2'-FL intake in non-breastfed infants and toddlers. This was similar to the intake estimation models presented in GRN 546 and GRN 571 (Glycom, 2014; Jennewein, 2015). Table 13 summarizes the estimated 2'-FL intake of non-breastfed infants and toddlers who consume non-exempt infant formula products. Mean intakes were observed to decrease as the infants' age increased, from 2.14 to 0.39 g per day (354 to 40 mg per kg bw per day), which is attributable to infants consuming a more varied diet at 6 months and older.

Table 13. Estimated Daily Intake of 2'-FL for Non-Breastfed Infants and Toddlers from Non-Exempt Infant Formula

Population Group	Age Group (months)	Consumer-Only Intake[1]					
		%	N	g/day		mg/kg bw/day	
				Mean	90th percentile	Mean	90th percentile
Infants	0-5	43.0	79	2.14	2.88*	354	498*
Infants	6-11	56.6	100	1.67	2.56	192	311
Toddlers	12-35	11.7	39	0.39	1.14*	40	101*

[1] These results represent the intake of 2'-FL from non-exempt infant formulas and follow-on formulas among consumers of formula (infants and toddlers reporting consuming breast milk in NHANES were omitted).
* The sample size does not meet the minimum reporting requirements; therefore, the intake estimate may not be statistically significant.

c. Medical Foods

In addition, Glycosyn and FrieslandCampina intend to incorporate 2'-FL at a maximum dosage level of up to 4 g per serving for use in medical foods for a target patient population of 11 years and older. The medical food use is recommended to consist of up to 3 servings per day. For certain chronic conditions, 2'-FL would be intended to be long term therapy, and will only be used as necessary under the guidance and direction of a physician. It should be noted that medical foods are used for the dietary management of a disease or condition, and therefore, the anticipated daily intake of 2'-FL would not be expected to be combined by a diet containing 2'-FL from conventional food uses. Therefore, the maximum anticipated daily intake from medical foods is 12 g per person per day (at the maximum dosage level of 4 g 2'-FL up to 3 times daily). These dosages are equivalent to 211 mg per kg bw per day for a 56.8 kg adolescent and 150 mg per kg bw per day for a 80.0 kg adult.

B. Estimated Dietary Exposure to Any Other Substance That is Expected to be Formed In or On Food

No other substances are expected to be formed in or on food under the intended conditions of use for Glycosyn and FrieslandCampina's 2'-FL preparation.

C. Dietary Exposure to Contaminants or Byproducts

There are no known concerns regarding dietary exposure to contaminants or byproducts of 2'-fucosyllactose.

PART 4. SELF-LIMITING LEVELS OF USE

There are no known self-limiting levels of use.

PART 5. EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

A. Other Information on Dietary Exposure

1. History of Human Food Use

The primary source of 2'-FL in the human diet is from human milk. The mean concentrations of 2'-FL in human milk range from 0.22 to 8.4 grams per L, depending on the genotype of the mother and stage of lactation, as indicated by the studies summarized in Table 14. Glycom (2016) summarized previous assessments of dietary intake of 2'-FL in human milk. Based on mean levels of 2'-FL present in mature human milk samples that have been reported in the literature, a 6.5-kg infant drinking 1 L of milk per day would be expected to consume 170 to 660 mg per kg body weight per day of 2'-FL. Among infants from secretor mothers, the intake of 2'-FL from mature breast milk may be up to 1,150 mg per kg body weight per day.

For newborn infants, the average intake of 2'-FL from colostrum is approximately 80 to 360 mg per kg body weight per day based on a 3.4-kg newborn infant drinking an average of 250 mL of breast milk per day during the first 5 days. However, in newborns from secretor mothers, the intake of 2'-FL from colostrum may be up to approximately 620 mg per kg body weight per day.

Table 14. 2'-FL Measured in Human Milk[a]

Sample	2'-FL (g/L)							Reference
	Not Specified	Day 1	Day 2	Day 3	Day 4	Day 5 - 10	> 10 days	
Average of 50 samples; stage unknown	2.5							Warren et al. (2001)
Average of 53 African (Burkinabe) women's colostrum		1.8	4.5	8.4				Musumeci et al. (2006)
Average of 50 Italian women's colostrum		1	2.1	4.2				Musumeci et al. (2006)
Average of 12 Samoan women						0.22	0.69	Leo et al. (2009)
Average of 42 Italian women					7.3	6.05	5.25	Gabrielli et al. (2011)
Average of 12 Japanese women		2.5	2	1.6				Asakuma et al. (2008)
Average of 11 US women		2.8	3	3.5			3.6	Chaturvedi et al. (2001a)
Average of 80 Asian women	2.1							Erney et al. (2000)
Average of 68 European women	2.6							Erney et al. (2000)
Average of 197 Latin-American women	2.5							Erney et al. (2000)
Average of 36 US women	2							Erney et al. (2000)
Average of 381 women (mixed geographies)	2.4							Erney et al. (2000)
Average of 19 women (mixed geographies)			2.8					Erney et al. (2000)
Average of 62 women (mixed geographies)					2.6			Erney et al. (2000)

Sample	2'-FL (g/L)							Reference
	Not Specified	Day 1	Day 2	Day 3	Day 4	Day 5 - 10	> 10 days	
Average of 300 women (mixed geographies)							2.25	Erney et al. (2000)
Unknown	0.45							Kunz et al. (1999) as reported in Castanys-Munoz et al. (2013)
Unknown					3.9		1.84	Coppa et al. (1999) as reported in Castanys-Munoz et al. (2013)
Unknown						0-3.45		Sumiyoshi et al. (2003) as reported in Castanys-Munoz et al. (2013)
Unknown						3.37	2.96	Thurl et al. (2010) as reported in Castanys-Munoz et al. (2013)

[a] Adapted, in part, from Castanys-Munoz et al. (2013).

2. Summary of Regulatory History of 2'-Fucosyllactose

a. US Regulatory History

In the US, the oligosaccharide 2'-FL has not been approved for use as a food additive. Based on available information from FDA's GRAS Notice Inventory website (FDA, 2017) as of August 19, 2017, three GRAS notice reviews (GRNs) were filed with FDA for 2'-FL and have received "no questions" letters from FDA. The details are listed in Table 15. In addition, there are a number of GRNs for other oligosaccharides that have received "no questions" letters from FDA as detailed in Table 16.

Table 15. FDA's GRAS Notice Inventory on 2'-Fucosyllactose[a]

Company/Reference	FDA GRAS Identifier	Material Identity	Intended Food Uses
1. Glycom A/S	GRN 546	2'-Fucosyllactose manufactured using chemical synthesis	In term infant formula (2.4 g/L); in a variety of foods and beverages (0.28 – 2.4 g/RACC)
2. Jennewein Biotechnologie GmbH	GRN 571	2'-fucosyllactose manufactured using a genetically engineered *E. coli*	As a nutrient for the body's nutritional and metabolic processes in term infant and toddler formulas (2 grams//Liter)

Company/Reference	FDA GRAS Identifier	Material Identity	Intended Food Uses
3. Glycom A/S	GRN 650	2'-Fucosyllactose manufactured using a genetically engineered *E. coli*	In term infant formula (2.4 g/L); in a variety of foods and beverages for babies and young children (0.084-2.04 g/serving)

[a] Glycosyn and FrieslandCampina's *E. coli* production strain has been modified in a different manner than those used by Jennewein and Glycom.

RACC – Reference amounts customarily consumed

Table 16. FDA's GRAS Notice Inventory for Oligosaccharides for use in Infant Formulas and Conventional Foods[a]

Company	FDA GRAS Identifier	Material Identity	Intended Food Uses
1. GTC Nutrition Company	GRN 44	Fructooligosaccharides (FOS)	Ingredient in conventional foods at various use levels (< 15.4%)
2. Mead Johnson	GRN 233	Galacto-oligosaccharides (GOS) in combination with polydextrose	Ingredient in milk-based infant formula (2 g/Liter)
3. Friesland Foods Domo	GRN 236	Galacto-oligosaccharides (GOS)	Ingredient in term infant formula (5g/L); In a variety of foods and beverages (1 g – 7.5 g/serving)
4. BioNeutra Inc.	GRN 246	Isomalto-oligosaccharide mixture	Use in a variety of foods, including meat product, at levels ranging from 1.5-15 g/serving
5. GTC Nutrition Company	GRN 285	Galacto-oligosaccharides (GOS)	Ingredient in baby, infant, and toddler foods (0.86-1.28 g/serving); in a variety of foods and beverages (1.28 g/serving)
6. GTC Nutrition Company	GRN 286	Galacto-oligosaccharides (GOS)	Ingredient in term infant formula and follow-on formula (7.2 g/L)

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
7. Yakult Pharmaceutical Industry Co., Ltd	GRN 334	Galacto-oligosaccharides (GOS)	Ingredient in term infant formula (7.2g/L); In a variety of foods and beverages (0.3 g – 9.5 g/serving)
8. Pfizer Nutrition and Beneo-Orafti	GRN 392	Oligofructose	Ingredient in milk-based term infant formula (3 g/L)
9. Shandong Longlive Bio-technology Co., Ltd.	GRN 458	Xylooligosaccharides	As a bulking agent in beers and ales (0.5 g/serving); in a variety of foods and beverages (0.2-2.4 g/serving)
10. Clasado, Inc.	GRN 484	Galacto-oligosaccharides (GOS)	Ingredient in a variety of foods and beverages (0.8-3.0 g/serving)
11. International Dairy Ingredients Inc.	GRN 489	Galacto-oligosaccharides (GOS)	Ingredient in term infant formula (4 g/L) and infant and toddler foods (3.8 mg/g); In a variety of foods and beverages (2.66 g/serving)
12. Clasado Inc.	GRN 495	Galacto-oligosaccharides (GOS)	Ingredient in term infant formula and follow-on formula (7.2 g/L)
13. New Francisco Biotechnology Corporation	GRN 518	Galacto-oligosaccharides (GOS)	Ingredient in a variety of foods and beverages (0.3-11 g/serving)
14. Ingredio, Inc.	GRN 537	Short-chain fructooligosaccharides (FOS)	Ingredient in infant formula and follow-on formula (400-500 mg/100mL)
15. New Francisco Biotechnology Corporation	GRN 569	Galacto-oligosaccharides (GOS)	Ingredient in term infant formula, and follow-on formula (7.2 g/L)
16. Nutricia North America, Inc.	GRN 576	Oligofructose and inulin	For use in exempt powdered term infant formula (<6.8 g/L)
17. Tata Chemicals Ltd.	GRN 605	Fructo-oligosaccharides (FOS)	Ingredient in infant foods (0-12 months) (0.4-6.7%)
18. Nestle Nutrition	GRN 620	Galacto-oligosaccharides (GOS)	Ingredient in non-exempt term infant and toddler formula (7.8 g/L)

COMPANY	FDA GRAS IDENTIFIER	MATERIAL IDENTITY	INTENDED FOOD USES
19. New Francisco Biotechnology Corporation	GRN 623	Fructooligosaccharides (FOS)	Ingredient in conventional foods, including infant foods, at levels ranging from 0.4-6.7%
20. BioNeutra North America Inc.	GRN 674	Isomalto-oligosaccharides	Ingredient in a variety of foods and beverages (5-100%)

[a] GRN 671, submitted by Vitalus Nutrition Inc. regarding Galacto-oligosaccharides (GOS), was filed by FDA, with an intended use in powdered non-exempt term infant formulas at levels not exceeding 7.2 g/L. At Vitalus Nutrition Inc.'s request, FDA ceased to evaluate the notification. GRN 717, submitted by Galam, Ltd. Regarding Short-chain fructo-oligosaccharides, with an intended use in a variety of foods and beverages (0.8-6.7%), was filed by FDA and is currently under review.

b. European Regulatory History

In the EU, the European Food Safety Authority (EFSA) reviewed the safety of 2'-FL as novel food ingredient. The EFSA panel concluded that 2'-FL is safe for infants (up to one year of age) when added to infant and follow-on formula, in combination with another oligosaccharide, lacto-N-neotetraose (LNnT), at concentrations up to 1.2 grams per L of 2'-FL and up to 0.6 grams per L of LNnT, at a ratio of 2:1 in the reconstituted formula. 2'-FL is also safe for young children (older than one year of age) when added to follow-on and young-child formula, at concentrations up to 1.2 grams per L of 2'-FL (alone or in combination with LNnT, at concentrations up to 0.6 grams per L, at a ratio of 2:1). The EFSA panel also concludes that 2'-FL is safe when added to other foods for adults, including dairy and milk products, dairy analogs, cereal bars, table top sweeteners, dietary foods for weight control diets, beverages and food supplements, at concentrations of 1.2 grams per L for beverage products, 1.2-2.4 grams per serving for food products and 3.0 grams per day for food supplements (EFSA, 2015). Following this EFSA review, 2'-FL in combination with LNnT was authorized as a novel ingredient by the European Commission for the intended uses as outlined in the EFSA review (EU, 2016).

PART 6. NARRATIVE

A. GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as:

> "...reasonable certainty in the minds of competent scientists that the substance is not harmful under the conditions of its intended use."[3]

[3] See 21 CFR 170.3 (e)(i) and 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 4/15/17).

Amplification is provided in that the conclusion of safety is to include probable consumption of the substance in question, the cumulative effect of the substance and appropriate safety factors. It is FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

> "...General recognition of safety requires common knowledge, throughout the expert scientific community knowledgeable about the safety of substances directly or indirectly added to food, that there is reasonable certainty that the substance is not harmful under the conditions of its intended use."
>
> "'Common knowledge' can be based on either 'scientific procedures' or on experience based on common use in food prior to January 1, 1958."[4]

FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following component elements:[5]

- Data and information relied upon to establish safety must be generally available, and this is most commonly established by utilizing published, peer-reviewed scientific journals; and
- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as JECFA and the National Academy of Sciences.

General recognition of safety based upon scientific procedures shall require the same quantity and quality of scientific evidence as is required to obtain approval of a food additive. General recognition of safety through scientific procedures shall be based upon the application of generally available and accepted scientific data, information, or methods, which ordinarily are published, as well as the application of scientific principles, and may be corroborated by the application of unpublished scientific data, information, or methods.

The apparent imprecision of the terms "appreciable," "at the time," and "reasonable certainty" demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Lu, 1988; Renwick, 1990; Rulis and Levitt, 2009).

[4] See 81 FR 54959 Available at: https://www.federalregister.gov/documents/2016/08/17/2016-19164/substances-generally-recognized-as-safe (Accessed on 4/15/17).
[5] See Footnote 1.

As noted below, this safety assessment to ascertain GRAS status for 2'-FL for the specified food uses meets FDA criteria for reasonable certainty of no harm by considering both the technical and common knowledge elements.

B. Glycosyn and FrieslandCampina's Findings on the Safety of 2'-Fucosyllactose

The literature search strategy that was employed to identify relevant information for assessing the safety of Glycosyn and FrieslandCampina's 2'-FL was based on the Chemical Abstract Service Registry Number (CASRN) and the common name "2'-Fucosyllactose" and used the TOXNET database, the RTECS database, and the natural products database NAPRALERTSM (NAtural PRoduct ALERT, Program for Collaborative Research in the Pharmaceutical Sciences, College of Pharmacy, University of Illinois at Chicago; www.napralert.org). There were five articles identified within the TOXLINE database for "fucosyllactose." Searches of the RTECS and NAPRALERTSM databases did not yield any results. A search was also conducted in PubMed for "2-Fucosyllactose" in order to identify relevant studies published through July 13, 2017. A Google search was also performed to identify relevant articles published in journals that are not indexed in PubMed.

Because 2'-FL is produced using fucosyltransferase produced by *E. coli*, information related to the safety of the organism used by Glycosyn and FrieslandCampina, *E. coli* K12 strain E997 (E638/pG217), was also sought. In this regard, the identification and taxonomic description of the bacterial strain, as well as precedents for its safe use in the context of human foods and drugs, were reviewed. The more significant information and publications are reviewed in the following sections.

1. Information Pertaining to Safety of *E. coli*

E. coli are bacteria that normally inhabit the intestinal tract of humans and other animals. Most strains are beneficial, but important pathogenic strains, although infrequently encountered, do exist and cause human illness. The background host organism that Glycosyn and FrieslandCampina use in their 2'-FL manufacturing process is the non-pathogenic strain *E. coli* K12. K12 is the standard "workhorse" organism used for most recombinant DNA work in laboratories worldwide. It has a defective cell envelope that renders it incapable of colonizing or surviving in the human gut. *E. coli* K12 contains no known pathogenic genes (either colonization factors or toxin genes) and is universally recognized as a safe, commercial manufacturing host. *E. coli* K12 is used globally in the commercial manufacturing of products ranging from amino acids and vitamins for foodstuff applications, to recombinant human proteins used in pharmaceutical applications, including protein products used as injectables. Pharmaceutical proteins expressed by *E. coli* include human insulin, growth hormones (somatostatin, somatotropin), immunomodulators (interferons, interleukins, TNFα), growth factors (G-CSF, EGF), blood factors, coagulation inhibitors (t-PA, staphylokinase), and enzymes (Schulze et al., 2006).

There is also precedent for the safe ingestion of live microbial *E. coli* preparations. Molecular genetic differentiation and identification methods make it possible to unequivocally distinguish pathogenic *E. coli* variants from non-pathogenic strains. Mutaflor® is an example of a probiotic therapy in which the active ingredient consists of a viable non-pathogenic *E. coli* strain, *E. coli* Nissle 1917. It is used in Europe and Canada for inflammatory and chronic functional bowel diseases (Mutaflor, 2016) (Mutaflor® website, https://aralez.com/Portfolio/mutaflor/). In the US, Mutaflor® was considered to be a "medical food," however, FDA classified Mutaflor® as a "biologic," and the product is currently discontinued from the US market pending a final decision (Mutaflor® website: https://aralez.com/Portfolio/mutaflor/). Probiotics are, by definition, living non-pathogenic micro-organisms that exert a positive effect on the host organisms when they enter the gastrointestinal tract in a viable condition in sufficiently large numbers (FAO/WHO, 2002). Mutaflor® has a 95-year record of being well tolerated and lacking adverse effects (Irrgang and Sonnenborn, 1988). In addition, non-pathogenic strains, unlike virulent *E. coli* variants, exhibit no harmful effects in toxicological studies in both conventional and germ-free animals (Schulze et al., 2006).

Glycosyn and FrieslandCampina conclude that the bioengineered organism is harmless to humans. This fact with the additional information of lack of genomic material and protein in the final product support the conclusion that the 2'-FL produced by this process is safe for human consumption.

2. Biological Activity of 2'-FL and Fucose-Containing Oligosaccharides

Human milk oligosaccharides (HMOs) comprise about 10% of the dry content of human milk and are a mix of more than 100 distinct molecular species made up of specific covalent configurations of five-component sugars: D-glucose, D-galactose, N-acetylglucosamine, *N*-acetylneuraminic acid, and L-fucose (Bode, 2012). The function of the oligosaccharide fraction of human milk was initially unknown, but work over the past three decades has revealed activities that can be categorized into three main areas: 1) prebiotic; 2) anti-infective; and 3) immunomodulatory, with activities in these areas often operating simultaneously. HMOs play an important role in the initial establishment and subsequent maintenance of a healthy infant gut microbiota, and in infant gut and immune system development. These beneficial activities have led to great interest in inclusion of these molecules in infant and adult foodstuffs.

The presence and relative abundance of α1,2- and α1,3- fucosylated species within the mixture of HMOs is determined by the status of a mother's "secretor" (FUT2, α1,2-fucosyltransferase) genes and her "Lewis" (FUT3, α1,3-, α1,4-fucosyltransferase) genes (Thurl et al., 2010). Common polymorphisms/mutations in FUT2 and FUT3 contribute to significant variations of milk oligosaccharide levels and milk oligosaccharide composition from individual to individual. Thus "secretor" (FUT2+) individuals (representing ~80% of mothers in most populations), are able to produce α1,2-linked fucose-containing structures in their milk, with 2'-FL (at levels ranging from 0.06 to 4.65 g per L) being the most abundant of these (Asakuma et al., 2008; Chaturvedi et al., 2001a), whereas "non-secretor" (FUT2-) individuals (representing ~20% of mothers in most

populations) produce no α1,2-linked fucose-containing structures in their milk. Moreover, even in the milk of "secretor" mothers, the concentrations of 2'-FL vary according to lactation stage (Chaturvedi et al., 2001a; Erney et al., 2000; Thurl et al., 2010).

a. Prebiotic Activities

Purified 2'-FL is an indigestible oligosaccharide that nevertheless can support the growth of beneficial bacteria in the colon, and a robust gut microbiota composed of commensal and mutualist bacteria can itself help to inhibit gut colonization by pathogens (Jandhyala et al., 2015). Some studies show that promoting the growth of specific gut bacterial populations can also reduce inflammation (Weiss et al., 2014).

The availability of purified 2'-FL has enabled numerous studies demonstrating the molecule's ability to support growth of commensal bacteria, both *in vitro* and *in vivo*. Yu et al. (2013a) showed that 2'-FL could stimulate the *in vitro* growth of two strains of the commensal *Bifidobacterium longum*, while an *E. coli* K12 strain and a *Clostridium perfringens* strain could not utilize 2'-FL for growth. In a subsequent study, Yu et al. (2013b) showed that 2'-FL stimulated the *in vitro* growth of 9 strains of *Bifidobacterium longum*, and one strain each of *Bifidobacterium infantis*, *Bacteroides vulgatus, Bacteroides fragilis,* and *Bacteroides thetaiotaomicron*. Strains of *Clostridium perfringens, Clostridium leptum, Lactobacillus rhamnosus, Enterococcus faecalis, Staphylococcus epidermidis, Enterobacter cloacae, Enterobacter aerogenes,* and *E. coli* did not grow on 2'-FL.

Sela et al. (2012) demonstrated that infant intestinal colonizers of the genus *Bifidobacteria* are highly adapted for utilizing certain fucosylated HMOs.

Bunesova et al. (2016) showed that three strains of *Bifidobacteria bifidum*, four strains of *Bifidobacterium longum*, and two strains of *Bifidobacterium kashiwanohensei* were able to utilize 2'-FL for growth *in vitro*.

Weiss et al. (2014) reported that supplementing the diet of newborn mice with 2'-FL led to a significant increase in the abundance of *Barnsiella* species (commensals included in the class *Bacteroidetes*) in the microbiota (60% of total microbiota under 2'-FL supplementation vs. 40% for unsupplemented controls). Importantly, 2'-FL supplementation also decreased the severity of induced colitis in dextran sulfate sodium (DSS)-treated mice relative to un-supplemented controls, and the magnitude of this difference correlated with *Barnsiella* abundance.

b. Anti-Infective Activities

Intestinal epithelium expresses the same set of glycosyltransferase genes as breast epithelium, and both intestinal mucin and intestinal epithelia are decorated with the same glycan structures that are found in HMOs. Thus, in principal, the soluble oligosaccharides found in human milk can directly contribute to protection of infants against enteric pathogens by acting as molecular "decoys," preventing the necessary first step in infection: pathogen binding to intestinal cells.

Campylobacter, a frequent cause of bacterial diarrhea that can lead to infant mortality, specifically binds to the H-1 antigen (Fucα1,2Galβ1,4GlcNAc) on gut epithelia. After identifying 2'-FL (which carries the very similar H-2 antigen, Fucα1,2Galβ1,4Glc) as capable of inhibiting *Campylobacter* adherence to its host cell receptor, Ruiz-Palacios et al. (2003) found that human milk contained sufficient fucosyloligosaccharide concentrations to inhibit *Campylobacter* binding and colonization *in vitro*, using an *ex vivo* human intestinal mucosa model. Human intestinal mucosa tissue was obtained from healthy ileum specimens of patients who required intestinal resection. The authors concluded that fucosyloligosaccharides serve as soluble ligands that compete with intestinal epithelial cell surface receptors for binding to pathogens that target α1,2 ligands.

Colonization and infection by *Campylobacter jejuni* in mice was then shown to be inhibited *in vivo* by administration *via* oral intubation of the neutral, fucosylated oligosaccharide fraction of human milk, which contains oligosaccharides carrying Fucα1,2Gal structures, principally 2'-FL (Ruiz-Palacios et al., 2003). BALB/c mice weighing 10-20 grams (6 per group, age and sex not specified) were assigned to one of the following treatment groups: 1) 2 mg of neutral milk oligosaccharides by oral intubation 2-hr before, during, and 2-hr after oral inoculation with low or high dose *C. jejuni* (total of 6 mg of oligosaccharides, equivalent to the amount in 1 mL of human milk, over 4 hours); 2) vehicle control (equivalent volumes of phosphate buffered saline before, during, and after the challenge with the same bacterial suspensions; or 3) a single 2 mg dose of neutral oligosaccharides to look for any adverse effects of their administration *per se*. Shedding of *Campylobacter* was determined by daily quantitative stool cultures for 5 days after the challenge. No effects were observed for the control groups at any time point. However, mice given neutral HMOs by oral intubation exhibited significantly less *Campylobacter* colonization at both low and high inocula. No ill effects on the mice were attributed to the ingestion of neutral milk oligosaccharides.

In a follow-up study, when H glycans (including 2'-FL) were expressed in the milk of transgenic mouse dams, it was shown that their nursing pups were strongly protected from infection by pathogenic *Campylobacter* (Ruiz-Palacios et al., 2003). Lactating mice carrying the FUT1 human fucosyltransferase gene expressed high levels of 2'-FL in their milk. The transgenic strain produced an average of 2.2 grams per L of milk compared with 0 grams per L in wildtype mice (Prieto et al., 1995). Dams of the transgenic strain and control wildtype dams were used in a subsequent study to suckle their pups in a *Campylobacter jejuni* colonization model. Suckling mice were orally inoculated with invasive strain 287ip and returned to their dams to continue nursing. Colonization was determined in one mouse from each of at least 10 litters every third day for 15 days. Intestinal clearance of *Campylobacter* was significantly greater in pups nursing from transgenic versus non-transgenic dams when the pups were challenged with identical inocula. No ill effects on the mice were attributed to the production or ingestion of 2'-FL; moreover, the clearance of *C. jejuni* in pups suckled on the transgenic milk was attributed to the presence of 2'-FL.

Furthermore, a clinical association study showed that *Campylobacter* induced diarrhea occurred less often in infants whose mother's milk contained high levels of 2'-FL (Morrow et al., 2004).

Yu et al. (2016) demonstrated that invasion of human epithelial HEp-2 and HT-29 cells by live *C. jejuni* was inhibited by 2'-FL at a concentration of 5 g per L, and that in these cultures that 2'-FL treatment suppressed the release of mucosal pro-inflammatory signals of interleukin IL-8 by 60-70%, IL-1b by 80-90%, and the neutrophil chemoattractant macrophage inflammatory protein 2 (MIP-2) by 50% ($P<0.05$). The authors then went on to show in an acute infection and recovery model performed in C57BL/6 mice, and designed to mimic the normal course of a human infection by *C. jejuni*, that administration of 2'-FL in drinking water at a concentration of 5 g per L reduced *C. jejuni* colonization by 80%, weight loss by 5%, histologic features of intestinal inflammation by 50-70%, and induction of inflammatory signaling molecules of the acute-phase mucosal immune response by 50-60% ($P<0.05$). No adverse events were reported in uninfected, 2'-FL treated control animals.

Cilieborg et al. (2017) showed that 2'-FL could reduce adhesion of a porcine pathologic enterotoxigenic *Escherichia coli* (ETEC) F18 strain to porcine jejunal epithelial PSIc1 cells at a dose of 5 g per L. A 2'-FL dose-escalating tolerability study performed in newborn piglets revealed no significant diarrhea over untreated control animals, or any reported negative impact of administered 2'-FL. The highest level of 2'-FL provided in the study in milk replacer (formula) was at a level of 10 g per L. However, 2'-FL in milk replacer at 10 g per L failed to prevent diarrhea in newborn piglets challenged with a very high dose of ETEC *E. coli* F18. Marginal improvements were reported for intestinal structure and function in the 2'-FL treatment groups. The very high dose of *E. coli* F18 utilized in the study may have masked any potential 2'-FL anti-infective benefits.

c. Immunomodulatory Activities

Several studies, including those of Yu et al. (2016) and Weiss et al. (2014) described above, have demonstrated that 2'-FL can modulate immune and inflammatory processes, producing effects through direct impacts on inflammatory pathways, or by indirect mechanisms mediated through the microbiota.

He et al. (2016) demonstrated that a mixture of HMOs containing 2'-FL, and also purified 2'-FL alone, can inhibit lipopolysaccharide (LPS)-mediated inflammation and IL-8 release during ETEC invasion of T84 and H4 intestinal epithelial cells *in vitro*. This is achieved through the direct attenuation of CD14 induction, although the precise mechanism of this attenuation is not known.

Goehring et al. (2016) performed a randomized, double-blind, controlled growth and tolerance study in healthy singleton infants comparing experimental formula that either contained 2'-FL (two doses, 0.2 g per L or 1 g per L) plus galactooligosaccharides (GOS), GOS alone, or were breastfed. Breastfed infants and infants fed either of the experimental formulas with 2'-FL were not different but had 29-83% lower concentrations of plasma inflammatory cytokines than did infants

fed the control formula containing no 2'-FL [interleukin receptor antagonist (IL-1ra), IL-1a, IL-1b, IL-6, and tumor necrosis factor-a (TNF-a); $P<0.05$].

Necrotizing enterocolitis (NEC) is a devastating and often fatal inflammatory condition found in premature and newborn infants characterized by the sudden onset of ischemia and necrosis in the small intestine or colon. The causes of NEC are not fully understood, although it is known that there is a genetic component (Morrow et al., 2011). Indications are that inadequate or inappropriate infant gut colonization may play an important role in the pathology. Inflammatory pathways are certainly involved. There are several reports that human milk is protective against NEC (Sullivan et al., 2010). Good et al. (2016) examined the impact of 2'-FL in a neonatal mouse model of necrotizing enterocolitis, and found that it protected against NEC, resulting in a decrease in pro-inflammatory markers, and that it helped preserve the small intestinal mucosal architecture. The authors observed changes in the microbiota on 2'-FL administration (dose: oral administration of 0.25 mg per g body weight once daily during the model), but attributed a significant part of the benefit they observed to the restoration of intestinal perfusion through up-regulation of the vasodilatory molecule endothelial nitric oxide synthase (eNOS). Administration of 2'-FL to eNOS-deficient mice, or to mice that received eNOS inhibitors, did not protect against NEC.

The neonatal pig from birth to age 3 weeks has been described as a good model for studying the gastrointestinal (GI) system of human infants up to age 3 months (Flamm, 2013; Formula, 2004; Guilloteau et al., 2010; Herfel et al., 2009; Odle et al., 2014) because they have similar specific digestive enzymes, nutrient absorption, gut closure, dietary requirements, microbial population, and gut transit time (Hanlon and Thorsrud, 2014). Cilieborg et al. (2016) used the porcine system to test whether infant formula enriched with 2'-FL would benefit gut microbial colonization and NEC resistance after preterm birth. Caesarean-delivered preterm pigs were fed formula (control, n=17) or formula with 5 g per L 2'-FL (2'-FL, n=16) for 5 days. Eight 2'-FL pigs (50%) versus twelve control pigs (71%) developed NEC, with no difference in lesion scores ($P=0.35$). This small reduction on NEC incidence on 2'-FL administration was not significant, although the authors did find a high inherent abundance of α1,2 fucosylated glycans in the intestinal mucosa of this neonatal pig cohort, perhaps providing a high bar to see impacts of the 2'-FL in the study. The 2'-FL at 5 g per L was otherwise well tolerated, and no deleterious 2'-FL impacts were noted.

Autran et al. (2016) investigated the efficacy of GOS on protection against NEC. Neonatal rats were randomly assigned to one of the following groups: dam-fed (DF), formula-fed (FF), FF containing pooled HMO (10 mg per mL), GOS (8 mg per mL), sialylated galactooligosaccharides (Sia-GOS) (500 µM), or 2'-FL (2 mg per mL), and then were subjected to a protocol to induce NEC. Supplementation with Sia-GOS or 2'-FL resulted in significant reductions in pathology scores. The DF and HMO fed groups had the lowest pathology scores, GOS did not have a protective effect, and the FF group had a significantly elevated pathology score.

Cilieborg et al. (2012) reported that provision of the optimal amount of enteral diets including mother's colostrum or milk can improve host defense by assisting the intestinal immune system in generating a beneficial response to bacterial colonization.

Hoeflinger et al. (2015) investigated the ability of individual strains of *Enterobacteriaceae* and an *Enterobacteriaceae* consortia enriched from piglet feces to grow on human milk oligosaccharides. They reported that neither the individual strains of *Enterobacteriaceae* nor the *Enterobacteriaceae* consortia enriched from piglet feces grew on 2'-FL, 6'-sialyllactose, or lacto-N-neotetraose (LNnT), but reported that some strains were able to utilize galactooligosaccharides, maltodextrin, and the mono- and disaccharide components of HMOs.

d. Other Activities

As a terminal sugar found on N- and O-linked glycoproteins, fucose-α(1−2)-galactose [Fucα(1−2)Gal] has been implicated in cognitive processes such as learning and memory. Both task-specific learning and long-term potentiation---an electrophysiological model of learning and memory---have been shown to induce protein fucosylation in hippocampal neurons (Murrey et al., 2009). Krug et al. (1994) studied the effect of 2′-FL on hippocampal long-term potentiation. Male Wistar rats were intrahippocampally injected with L-fucose and the sugar epitope 2′-fucosyllactose prior to induction of long-term potentiation (LTP). 2′-FL had only a minimal and short-lasting depressive effect on the monosynaptically evoked field potential recorded in the dorsal blade of the dentate gyrus of freely moving rats upon stimulation of the perforant pathway. However, LTP induced by fractionated tetanization of the perforant pathway was sustained at the initial level 48 hours after tetanization while it declined within 24 hours in control animals injected with lactose ($P < 0.01$). 2′-FL also enhanced hippocampal LTP *in vitro* (Matthies et al., 1996). The results support earlier findings that have indicated a participation of fucosylated macromolecules in the maintenance of LTP. However, because Krug et al. (1994) employed a parenteral route of administration, the study was not considered further here. Oliveros et al. (2016) reported that compared with controls, treatment of rats with 2'-FL for one year resulted in significantly improved performance in the Novel Object Recognition and Y maze paradigms, and LTP was more intense and longer lasting in the rats supplemented with 2'-FL in young and adult animals.

e. Summary

Glycosyn and FrieslandCampina have reviewed these studies and none of these biological activities for 2'-FL raise any safety concerns for proposed uses.

3. Absorption, Distribution, Metabolism and Excretion of 2'-FL

2′-FL is among the human milk oligosaccharides (HMOs) that are considered to be "dietary fiber" because they are poorly absorbed by the human gut (Engfer et al., 2000; Gnoth et al., 2000). This was demonstrated in a study that used semi-quantitative methodology (Chaturvedi et al., 2001b). As a non-digestible sugar, 2′-FL is available to act as a carbon source (a prebiotic) for commensal organisms in the lower intestine. Specifically, 2′-FL supports the growth of a variety of beneficial bacteria *in vitro*, including *Bifidobacteria* and *Bacteroides* species (Newburg, 2009).

2′-FL undergoes partial fermentation in the colon when infants are given a load of HMOs (a purified oligosaccharide fraction from mothers' milk) (Brand Miller et al., 1995; Brand-Miller et al., 1998).

Despite the fact that HMOs are non-digestible, it has been shown that some HMOs can be absorbed intact and enter the circulation (Goehring et al., 2014). Chaturvedi et al. (2001b) compared the profile of HMOs in the feces and the urine of infants that were fed mothers' milk to the profile in the feces and urine of infants that were fed formula. They reported that oligosaccharide concentrations in the feces were higher than those in mothers' milk and much higher than that in urine. According to Goehring et al. (2014), 2'-FL and other human milk oligosaccharides have been identified in the urine and plasma of breast-fed infants at levels that correspond to the amounts in human milk, but not formula-fed infants. They also reported that 2'-FL was not present in the circulation of infants who consumed breast milk that did not contain 2'-FL. Coppa et al. (2001) reported that 40-50% of HMO is present in the feces of breast-fed infants.

Multiple studies have shown that administration of a bolus of ^{13}C-galactose or ^{13}C-glucose to lactating mothers results in the presence of ^{13}C-HMO in the urine of infants (Dotz et al., 2014; Obermeier et al., 1999; Rudloff and Kunz, 2012). Gnoth et al. (2001) showed that *in vitro* transport of neutral HMOs such as 2'-FL across the intestinal epithelium occurs *via* receptor-mediated transcytosis and paracellular pathways (Vazquez et al., 2017).

Altogether, studies show that a minor portion of HMOs (and 2'-FL) is absorbed into the circulation, whereas the majority of HMOs (and 2'-FL) are not absorbed and function as a substrate for the growth of the intestinal microbiota.

4. Toxicology Studies on 2'-FL

Published and unpublished conventional toxicology studies on various sources of 2'-FL are described below. The absence of toxicity with very high no observed adverse effect levels (NOAELs) was demonstrated in several subchronic studies in rats on test material consisting of 2'-FL from various sources. A comparison of the chemical composition of the test materials is shown in Table 17.

Table 17. Comparison of 2'-FL Samples used in Toxicological Studies

Parameter	2'-FL Specification			
	Glycom A/S GRN 546; Coulet et al. (2014)	Jennewein Biotechnologie GRN 571;Hanlon and Thorsrud (2014)	Glycom A/S GRN 650	Glycosyn/ FrieslandCampina
Test article purity	99%	94.1%	97.6%	94.0%
Manufacturing process	Chemical synthesis	Fermentation	Fermentation	Fermentation
Production organism starter strain	--	*E. coli* BL21 (DE3)	*E. coli* K12 (DH1/ ATCC 33849)	*E. coli* K12 (GI724/ ATCC 55151)
Specification				
Assay by HPLC	Min. 95.0%	≥ 90%[a]	Min. 94.0%	Min. 90%
D-Lactose	Max. 3.0 w/w%	≤ 5%[a]	Max. 3.0 w/w%	Max. 3%
Allo-lactose	NS	NS	NS	Max. 2%
L-Fucose	Max. 1.0 w/w%	≤ 3%[a]	Max. 1.0 w/w%	Max. 2%
Galactose	NS	≤ 3%[a]	NS	Max. 2%
Glucose	NS	≤ 3%[a]	NS	Max. 2%
Difucosyllactose	Max. 1.0 w/w%	≤ 5%[a]	Max. 1.0 w/w%	NS
2'-Fucosyl-D-lactulose	Max. 0.6 w/w%	NS	Max. 1.0 w/w%	NS
Fucosylgalactose	NS	≤ 3%[a]	NS	NS
3-Fucosyllactose	NS	≤ 5%[a]	NS	NS
Protein	0.1%	≤ 100 ppm	0.01%	Max. 0.01%
Ash	0.2%	≤ 0.5%	Max. 1.5%	Max. 0.2%

[a] Percent of total carbohydrates by HPLC
NS Not specified

a. Published Toxicology Studies on 2'-FL

A search of the TOXNET database was conducted on July 12, 2017. Several toxicology studies have been conducted on 2'-FL.

i. Studies in Rats

Coulet et al. (2014) conducted a 14-day tolerability and dose-range finding study using 7-day-old [post-natal day (PND) 7] Wistar [Crl:WI(Han)] rats. Five animals per sex per group were administered Glycom's 2'-FL (purity of 99%) by gavage at doses of 0 (vehicle control), 2,000, 5,000, or 7,500 mg per kg body weight (bw) per day. A reference control group was administered 7,500 mg oligofructose (OF) per kg bw per day during the 14-day study. Observations were conducted two times per day for general health, mortality, and morbidity, clinical observations were conducted once per day, and detailed clinical examinations were conducted once per week. Body weights were measured on post-natal days 1, 4, 7, 10, 14, 17, and 20. All animals were euthanized at the end of the 14-day administration period and macroscopic examinations were performed. One female in the 7,500 mg per kg bw per day group died on day 12, with no significant findings at necropsy. One female rat that was partially cannibalized on day 6 in the 7,500 mg per kg bw per day group had presented clinical signs and lost body weight on days 0 to 3. No compound-related macroscopic findings were observed at necropsy. The cause of death was undetermined. In the 7,500 mg per kg bw per day group, the OF control group, and to a lesser extent, the 5,000 mg per kg bw per day group, liquid and/or yellowish liquid feces were observed in some animals from days 1 to 3 up to days 9 to 11, and were occasionally observed in conjunction with erythema in the urogenital region. All animals in the 5,000 and 7,500 mg per kg bw per day groups, and in the OF control group, had lower body weight gains between days 0 to 3 as compared with the vehicle control group. The authors concluded that the highest suitable dose of 2'-FL for the 90-day study that followed was lower than 7,500 mg per kg body weight per day and therefore set a high dose of 6,000 mg per kg per body per day in the subchronic toxicity study that followed.

A 90-day subchronic oral toxicity study of 2'-FL with a 4-week recovery period was conducted starting with 7-day-old Wistar [Crl:WI(Han)] rats (Coulet et al., 2014). The period of administration of Glycom's 2'-FL occurred within the window of time when immune and sexual maturity take place in rats. 2'-FL (purity of 99%) was administered at doses of 2,000 and 5,000 (n=10 animals per sex per dose group), and 6,000 mg per kg bw per day (n=15 animals per sex). The vehicle (water) control group (0 mg per kg bw of 2'-FL per day) consisted of 15 animals per sex. A reference control group was administered 6,000 mg per kg bw per day of OF (15 animals per sex). Standard diet (A04C-10) and water were provided *ad libitum*. Clinical observations were conducted once per day, observations were conducted twice per day for mortality and morbidity, and detailed clinical examinations were conducted once per week. Body weights were measured prior to dosing and twice weekly during the first 8 weeks of the study and then once per week for the remainder of the study. Food intake was measured twice weekly starting at week 2 until week 8, and then once per week for the remainder of the study. During the last week of administration, ophthalmological analyses were conducted on animals from the control group, the 6,000 mg per kg bw per day 2'-FL

group, and the 6,000 mg per kg bw per day OF group. Hematology, coagulation, clinical chemistry, and urinary analyses were conducted at the end of the administration period. Twenty animals from each treatment group (10 rats per sex) were euthanized and necropsied. The remaining animals, 5 rats per sex per group in the vehicle control, the 6,000 mg per kg bw per day 2'-FL group, and the reference control group of OF were observed for 4 weeks, after which all animals were euthanized, necropsies were performed, and histopathological analyses were conducted on all organs and tissues. Kidneys of all females in the 2,000 and 5,000 mg per kg bw per day groups and in all recovery groups were microscopically inspected. Clinical pathology was performed on all animals from all groups.

One male and one female rat in the 6,000 mg per kg bw per day 2'-FL dose group, two males and one female in the 6,000 mg per kg bw per day OF dose group died during the treatment period. One female in the 6,000 mg per kg bw per day OF group died during the recovery period. The authors stated that because there was no histopathological correlation to their deaths, they could not show a relationship to treatment. Diarrhea occurred occasionally for rats of both sexes in the 2,000 mg per kg bw per day dose group and for all animals in the 5,000 and 6,000 mg per kg bw per day of 2'-FL treatment groups and the OF treatment group. The authors noted that this effect was associated with erythema in rats that were treated with 6,000 mg per kg bw per day of 2'-FL and OF. Rats of both sexes also experienced hyper salivation. There were no significant differences in food consumption or terminal body weights between any test group and the control group during the treatment or the recovery period. No compound-related ophthalmological findings were reported. Occasional significant changes in hematological parameters in female rats were attributed to low grade chronic stress related to diarrhea. A significant increase in prothrombin time for males in the 6,000 mg per kg bw per day dose group was described as slight and unrelated to the test article. In clinical chemistry analyses, significant reductions in aspartate aminotransferase in rats of both sexes in the 6,000 mg per kg bw per day 2'-FL and OF dose group and in the 5,000 mg per kg bw per day dose groups were not considered by the authors to reflect an adverse event. Other changes were of low magnitude, typically remained within the range of historical control values, and occurred in a single sex. Urinalysis revealed a significant reduction in specific gravity that appeared to be dose-dependent; however, the authors described the magnitude of the change as too small to be considered toxicologically relevant.

There were statistically significant decreases in absolute adrenal weights in males in the 5,000 and 6,000 mg per kg bw per day groups, the relative adrenal weights of males in the 6,000 mg per kg bw per day group, and in absolute brain and relative kidney weights in females in the 6,000 mg per kg bw per day group. There were statistically significant increases in heart weights in males in the 5,000 mg per kg bw per day group. These differences in organ weights were not associated with histological changes, did not occur in both sexes, had resolved by the end of the 90-day treatment period, or also occurred in control group animals. Therefore, differences in organ weights were not considered to be toxicologically significant. The animals that died during the treatment period had reduced lymphoid follicle development of the spleen. At the end of the administration period, females in the 5,000 mg per kg bw per day and 6,000 mg per kg bw per day dose groups and in

the OF group exhibited an elevated incidence of minimal cortical tubular epithelial cytoplasmic vacuolation of the kidneys in the absence of renal degeneration. Similar effects were observed in females in the control group at the end of the recovery period. The authors considered these effects to be non-adverse. Because an association between the treatment and the deaths of two animals in the 6,000 mg per kg bw per day dose group could not be excluded, the authors concluded that the no-observed-adverse-effect level (NOAEL) for 2'-FL was 5,000 mg per kg bw per day in Wistar [Crl:WI(Han)] rats.

Glycosyn and FrieslandCampina agree with the authors' conclusions on the results of this subchronic study. The test material used in this study is sufficiently similar to the specifications of the Glycosyn and FrieslandCampina 2'-FL to support the safety of the latter. Glycosyn and FrieslandCampina believe that the NOAEL of 5,000 mg per kg bw per day is a sufficient margin of safety to conclude that the exposure levels for all age groups estimated in Part 3.A of this notice is safe.

ii. Studies in Pigs

Twenty-seven male and 21 female domestic farm piglets (Domestic Yorkshire Crossbred Swine) were employed in a 20-day oral toxicity study on Jennewein 2'-FL (Hanlon and Thorsrud, 2014). The 2'-FL used in this study was manufactured via a fermentation process. The pigs were fed a liquid diet that included 0 (vehicle control), 200, 500, or 2,000 mg Jennewein 2'-FL per L starting when they were two days old. The diets that included 2'-FL were equivalent to 29.37, 72.22 and 291.74 mg per kg per day 2'-FL, respectively, in males and 29.30, 74.31, and 298.99 mg per kg per day 2'-FL, respectively in females. The piglets were checked twice daily for signs of morbidity, mortality, and injury, clinical examinations were conducted two times per week, and blood samples were taken on study days 7 and 21. Body weights were measured daily during the first week and every other day during the remainder of the study. The animals were sacrificed on Day 22. At necropsy, organ weights were measured and histopathological examinations were conducted on the brain, heart, kidneys, large intestine (cecum, colon, rectum), liver, small intestine (duodenum, jejunum, ileum), spleen, eyes, gall bladder, stomach, lung with bronchi, mesenteric lymph nodes, pancreas, and Peyer's patches. Watery feces were observed in 5 animals in the 2,000 mg per dL dose group (3 males and 2 females), 3 animals (1 male and 2 females) in the 500 mg per L dosing group and 4 animals (2 males and 2 females) in the 200 mg per L dosing group. The authors stated that these results were not dose-related. A reduction in appetite was observed in one male and two females in the 2,000 mg per L dose group for one day and for one female in the 200 mg per L dose group for two days, but was not dose related. Adverse effects were observed, including elevated alanine aminotransferase (ALT) levels in males in the 2,000 mg per dL dose group. However, because they were not considered to be dose-related and occurred in the absence of other related pathological effects, these adverse effects were not considered by the authors to be toxicologically significant. The authors reported that no adverse effects associated with Jennewein 2'-FL were observed on growth and development, clinical pathology, and histopathology at terminal necropsy. The authors concluded that the administration of Jennewein 2'-FL in Purina ProNurse®

milk replacement formula to neonatal piglets, from birth to age 3 weeks, at concentrations of up to 2,000 mg 2'-FL per L per day was well tolerated by piglets.

Based on the data presented, Glycosyn and FrieslandCampina agree that Jennewein's 2'-FL was well tolerated by neonatal pigs at a dose of 2,000 mg 2'-FL per day and that this study supports the conclusion of safety for the proposed uses of 2'-FL especially for infants and toddlers.

iii. Genotoxicity Studies

Coulet et al. (2014) conducted a bacterial reverse mutation assay (Ames test) on Glycom's 2'-FL (purity of 99%) using test strains TA98, TA100, TA1535, TA1537, and TA102 of *Salmonella typhimurium* in the presence or absence of metabolic activation (S9). The study adhered to Good Laboratory Practice principles (OECD, 1998a) and was conducted in accordance with OECD (1997a). Bacterial strains were incubated at concentrations of 52, 164, 512, 1,600, or 5,000 µg 2'-FL per plate when the plate incorporation method was used. In the pre-incubation method experiment, bacterial strains were incubated at concentrations of 492, 878, 1,568, 2,800, or 5,000 µg 2'-FL per plate. Water was used as the vehicle control. In assays conducted in the absence of S9, 2-nitrofluorene was used as the negative control for strain TA98, sodium azide was used as the negative control for strains TA100 and TA1535, 9-aminoacridine was used as the negative control for strain TA1537, and t-butyl hydroperoxide was used as the negative control for strain TA102. In assays conducted in the presence of S9, 2-aminoathracene was used as the positive control. There was no biologically significant increase in the number of revertant colonies in the treatment with 2'-FL compared with the negative control at any concentration either in the presence or absence of S9 in both the plate incorporation and the pre-incubation methods. There were increases in the number of revertant colonies in the treatments with positive control agents. There was no cytotoxicity or precipitation observed in any strain treated with 2'-FL in the presence or absence of S9. Thus, 2'-FL was determined to be non-mutagenic in the Ames test at concentrations up to 5,000 µg per plate.

Coulet et al. (2014) investigated the mutagenic potential of Glycom's 2'-FL (99% purity) in an *in vitro* mammalian cell gene mutation test in L5178Y tk+/- mouse lymphoma cells. The study was conducted in accordance with OECD Test Guideline 476 (OECD, 1997b) and adhered to Good Laboratory Practice principles (OECD, 1998a). Cells were incubated for 24 hours with 2'-FL at concentrations ranging from 1.7 to 5,000 µg per mL in the absence of S9. In a separate experiment, cells were treated for 4 hours with concentrations of 2'-FL ranging from 492 to 5,000 µg per mL in the absence or presence of S9. There was no evidence of precipitation or cytotoxicity at any dose of 2'-FL and there were no statistically or biologically significant increases in the frequency of mutations in cells treated with 2'-FL in the presence or absence of metabolic activation. The authors concluded that 2'-FL showed no mutagenicity at doses of up to 5,000 µg per mL under the conditions described.

iv. Summary

Glycosyn and FrieslandCampina concluded that the published *in vitro* toxicity studies (*Salmonella* mutagencity assay and mouse lymphoma assay) on Glycom's 2'-FL showed no mutagenic activity and indicate that there is no likely carcinogenic risk from consumption of 2'-FL. Glycosyn and FrieslandCampina believe that the specifications for their 2'-FL is sufficiently similar to these published studies and supports the safety of their 2'-FL.

b. Unpublished Toxicology Studies on the Subject 2'-FL

FrieslandCampina conducted a number of toxicology studies on the subject 2'-FL. The results of these studies confirmed the results of previously published studies and are considered corroborative to the published safety evidence. It should be noted that FrieslandCampina is preparing a manuscript for the publication of these studies. When the publications become available, they will enhance the publicly available evidence documenting the safety of 2'-FL for human consumption. A summary of these studies follow and copies of the complete laboratory reports of these studies are included as Appendices 9-12.

i. Studies in Rats

FrieslandCampina contracted Triskelion Laboratories to conduct a 90-day dietary study on the subject 2'-FL in Wistar outbred (Crl:WI(Han)) rats according to OECD guidelines. Doses of 2'-FL for this study were selected by conducting a 14-day range finding study in male rats. In the range finding study, 2'-FL was added at 0, 3, 6, or 10% of the diet. The doses of 2'-FL calculated from food consumption data in this study were 0, 2.56, 5.08, and 7.99 g per kg bw.

No treatment related effects were seen on clinical signs, body weights, food consumption, and macroscopic examination. Organ weights were normal with two exceptions. Relative liver weights were decreased in the mid- and high-dose groups. Elevations of absolute and relative cecal weights were observed in the mid- and high dose groups. The investigators concluded that decreases in liver weights are not usually considered toxicologically significant and the increase in cecal weights were likely a physiological adaptation to the test material as studies on other poorly digestible and fermentable sugars showed similar effects and this effect was not considered to be adverse in those studies (WHO, 1987). Based on these results, a decision was made to use the same treatment levels of 2-FL in the 90-day rat study. A complete copy of the report on the range finding study is included as Appendix 9.

The 90-day study was conducted with the same doses of 2'-FL in the same species, using groups of 10 rats of each sex per dose group. All animals survived to the end of the study with the exception of one mid-dose female whose death was considered not related to treatment. Sporadic and slight (<10% compared to control) increases in water consumption and decreases in food consumption at some measurement points during the study were not considered to be related to treatment. Based on weekly food consumption measurements, these dietary levels provided an overall mean intake of the test substance in the low-, mid- and high-dose groups of 2.17, 4.27, and

7.25 g per kg bw per day for males and 2.45, 5.22, and 7.76 g per kg bw per day for females, respectively. Analyses of homogeneity, content, and stability of the test substance in the test diets confirmed that the rats consumed the intended amounts of the test substance. Clinical signs were considered normal for all animals. Neurobehavioral observations and motor activity assessments in a functional observational battery did not indicate any neurotoxic potential of the test substance. Ophthalmoscopy did not reveal any treatment related ocular changes.

Hematological and clinical chemistry analyses were conducted on all rats at necropsy. There were no toxicologically significant changes in red blood cell variables or in total and differential white blood cell counts. The investigators discounted an increase in thrombocytes in high-dose females because it was slight and not seen in males. There were no treatment related changes in any clinical chemistry measurements. An increase in the urea concentration in mid-dose and high-dose males was considered a chance finding in the absence of this finding in females and any corroborative histopathological findings in males. No significant changes were seen in urinalysis measurements.

The relative weight of the liver was slightly (approximately 8%), but statistically significantly increased in males in the high-dose group. This elevated relative liver weight was not accompanied by changes in clinical chemistry or microscopy of the liver and did not occur in females and was therefore not considered to be adverse.

Significantly increased cecal weights were seen in males at all doses and in mid- and high-dose males. Histopathology of the cecum was considered normal in all high dose animals. The investigators concluded that the effect on the cecum was due to physiological adaptation to the nature of the test materials being an indigestible carbohydrate. This effect has been well documented in the literature as an adaptive effect (WHO, 1987) and has been seen in studies with hydroxypropyl starches (Leegwater et al., 1974), fructans (Demigne et al., 2008) and cellulose and glucomannan (Oku, 1995). In a more recent rat study, several forms of dietary fiber were found to increase cecal weight and improve the histomorphology of the cecal lumen. The investigators concluded that increased crypt depth as a result of dietary supplementation of low-digestible carbohydrates is a beneficial morphological effect. The crypts contain intestinal stem cells, the principal site of cell proliferation in the intestinal mucosa, and increased depth is associated with increased rate of turnover of intestinal mucosal cells (Knapp et al., 2013).

Macroscopic examination at necropsy and microscopic examination of organs and tissues did not reveal treatment-related findings.

It was concluded that the subject 2'-FL did not induce any toxicologically relevant changes in any test group, and therefore, the authors of the study decided that the NOAEL was the highest level tested, i.e., 10 % in the diet (≥ 7.25 g per kg body weight per day). FrieslandCampina and Glycosyn conclude that the results of this study are corroborative to published studies that have supported previous conclusions that 2'- FL can be added in the human diet. A complete copy of the study report is attached as Appendix 10.

ii. Genotoxicity Studies

FrieslandCampina also contracted Triskelion Laboratories to conduct two *in vitro* genotoxicity studies.

In a bacterial mutagenicity study, no significant increase in mutations was observed in a single assay in four tester strains of *Salmonella* (TA 98, TA 100, TA 1535 and TA 1537) and one strain of *E. coli* (WP2 *uvrA*) in the absence or presence of an exogenous liver extract (S9) to provide metabolic activation with five concentrations of test material up to a concentration of 5,000 µg per plate. No toxicity was observed in any strain at any dose. A complete copy of this study report is attached as Appendix 11.

A second study was conducted in cultured binucleated human lymphocytes. In an experiment with pulse treatment with and without metabolic activation, marginal cytotoxicity and no increase in the occurrence of micronuclei were observed at 3 concentrations (500, 1,000 and 2,000 µg per mL). In a second experiment with continuous treatment at the same concentrations without metabolic activation, marginal cytotoxicity and no increase in the occurrence of micronucleated cells was seen. The investigators concluded that 2'-FL did not exhibit any clastogenic or aneugenic activity in this cell system. A complete copy of the study report is attached as Appendix 12.

FrieslandCampina and Glycosyn conclude that the results of these studies corroborate information in the published literature that indicate that 2'- FL has no known genotoxic effect and likely presents no carcinogenic risk to humans.

c. Other Unpublished Studies on 2'-FL

i. Studies in Rats

In an unpublished, oral toxicity pilot study that was conducted in accordance with OECD guideline 408 (OECD, 1998b), 10 female rats (Crl:CD(SD)) were fed a standard rat diet (control) or the same diet that included 10% Jennewein 2'-FL *ad libitum* for 7 days. This study was discussed in GRN 571 (Jennewein, 2015). There were no deaths of animals, significant differences in food consumption or body weight between control and treatment groups, nor were there any behavior changes or changes in appearance.

GRN 571 describes a 90-day study (unpublished) in which a total of 40 male and female CD® rats (Crl:CD(SD)) were fed a standard rat diet (ssniff-R/M-H V1530) *ad libitum* (control) or the standard rat diet that was supplemented with 10% of Jennewein's 2'-FL (10 rats per sex per dose group) (Jennewein, 2015). An additional 3 animals per sex in the control group and nine animals per sex in the treatment group were used exclusively for blood sampling. None of the animals died during the study. There were no differences in food consumption, body weight, or body weight gain in males or females in the treatment group compared with the control group. There were no differences between treatment and control groups in clinical signs, food consumption, body weight, behavior, appearance, hematology, clinical biochemistry, urinalysis, or ophthalmological

examination. Intake of 2'-FL decreased during the study from 11.54 g per kg per day to 5.25 g per kg per day in male rats (mean=7.66±2.21 g per kg per day) and from 12.07 g per kg per day to 5.78 g per kg per day in female rats. At necropsy, there were no differences in organ weights, gross pathology, or histopathology between the treatment group and the control group. The authors stated that histopathological effects were not treatment-related. Pale stools were observed in 7 of 10 males and 4 of 10 females between days 9 and 69 of the study in the 2'-FL group. This effect was attributed to the amount of undigested test item in the feces and was not considered by the authors to be adverse. In addition, one male rat had soft stools starting on day 14 for a 15-day period. This effect was not thought to be related to 2'-FL consumption. The study authors concluded that Jennewein's 2'-FL was safe at average doses of 7.66 grams per kg per day and 8.72 grams per kg per day (the NOAEL) in female and male rats, respectively. Glycosyn and FrieslandCampina agree that this determination of the NOAEL is appropriate.

As described in GRN 650, Penard (2015a) conducted a 90-day oral toxicity study with an additional 28 day recovery period in Wistar [Crl:WI(Han)] rats on Glycom's 2'-FL (Glycom, 2016). The study was conducted in accordance with OECD standard of Good Laboratory Practice. In the main study seven-day old neonatal Wistar rats were administered 2,000, 4,000, or 5,000 mg per kg bw of Glycom's 2'-FL (produced by fermentation, purity 97.6%) or 5,000 mg per kg bw per day of FOS (reference group) for 90 days. Animals in the recovery group 5 (rats per sex) were also administered control, 2'-FL or FOS for 90 days after which they remained untreated for 28 days and were killed after the 90-day time period. One dam was then housed with a reconstituted litter of 5 pups per sex, fed a standard diet (A04C-10), and the pups were treated with the same dose of 2'-FL until weaning on day PND 21. No deaths of animals that were associated with the test item occurred. Liquid feces were noted for most rats that were treated with FOS and for animals in the mid- and high dose 2'-FL groups. Rats that were treated with the mid-dose of 2'-FL also had soiled urogenital regions. Beginning on day 35 of the main part of the study, the rats treated with FOS or the mid- or high- doses of 2'-FL showed hypersalivation, abnormal foraging, and/or pedaling, but this effect was not observed during the recovery period.

There were no ophthalmological effects related to test article administration observed and there were no remarkable effects on body weight, body weight gain, or food consumption. There were no toxicologically relevant changes in tibia length, reflex and physical development, time to sexual maturation, learning capacity, memory, motor activity (Morris water maze, exploratory behavior, or general movement (open-field test). Small differences in hematological parameters were not considered to be toxicologically significant. There were some significant changes in serum chemistry parameters including reductions in triglyceride concentrations for the mid and high dose 2'-FL groups in comparison to the control and reference groups and reduced concentrations of cholesterol in all males given 2'-FL and in females that were give the mid and high doses of 2'-FL. These changes were small and or remained within the normal historical control data range and did not occur during the recovery period. As a result, the study investigators concluded that these effects were not adverse. No differences in urinalysis, organ weights, macroscopic or histological

observations were attributable to treatment with 2'-FL. The authors determined a NOAEL of 5,000 mg per kg bw per day.

Glycosyn and FrieslandCampina agree that this determination of the NOAEL (5,000 mg per kg bw per day) is supported by the results of this study and that the results of this unpublished study provide corroborative evidence for the safety of 2'-FL.

ii. Genotoxicity Studies

In an unpublished study described in GRN 571 (Jennewein, 2015), the mutagenicity of Jennewein's 2'-FL at concentrations of up to 5,000 μg per plate was tested in a bacterial reverse mutation test using *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535, and TA 1537 in the absence and presence of metabolic activation (S9). The plate incorporation and pre-incubation methods were used and the study was conducted in accordance with OECD Test guideline 471 (OECD, 1997a) and adhered to current Good Laboratory Practice. There were no signs of cytotoxicity, nor were there any increases in the numbers of revertant colonies in any of the five test strains with or without activation. Significant increases in the number of revertant colonies were observed for the positive controls. The authors concluded that Jennewein's 2'-FL was not cytotoxic or mutagenic at doses of up to 5,000 μg per plate under the conditions of this study.

Glycosyn and FrieslandCampina agree that there was no evidence of mutagenicity or cytotoxicity of Jennewein's 2'-FL at doses of up to 5,000 μg per plate.

The mutagenicity of Glycom's 2'-FL produced by fermentation (HPLC purity=97.6%) was investigated in the *Salmonella* mutagenicity assay (Verspeek-Rip, 2015a). As described in GRN 650, *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA 1537 and *E. coli* strain WP uvrA were exposed to 2'-FL in the presence or absence of metabolic activation. For the plate incorporation method, the doses of 2'-FL used were 52, 164, 512, 1,600, or 5,000 μg per plate and for the preincubation method, the doses used were 492, 878, 1,568, 2,800, or 5,000 μg per plate. No cytotoxicity or precipitation occurred at any dose in any bacterial strain in the presence or absence of metabolic activation and there was no biologically significant increase in the number of revertant colonies. It was concluded that Glycom's 2'-FL produced by fermentation was not cytotoxic or mutagenic under the conditions of this study and provides corroborative evidence of the safety of 2'-FL.

An *in vitro* micronucleus test conducted on concentrations of up to 2,000 μg per mL of 2'-FL manufactured by chemical synthesis was described in GRN 650 (Glycom, 2016). The study was conducted in accordance with OECD Guidelines 487 (OECD, 2014) and followed Good Laboratory Practice principles (OECD, 1998a). There was no significant increase in the number of micronucleated peripheral human lymphocytes in the presence of absence of metabolic activation (Verbaan, 2015a).

The EFSA Panel on Dietetic Products, Nutrition, and Allergies (EFSA, 2015) reviewed an unpublished *in vitro* micronucleus assay on 2'-FL produced by fermentation in the presence and absence of metabolic activation that was submitted by Glycom A/S as part of their data package when EFSA asked to deliver an opinion on 2'-*O*-fucosyllactose as a novel food ingredient (Unpublished Study Report, 2015) cited in GRN 650 as Verbaan (2015b). The study was conducted in accordance with OECD Guideline 487 (OECD, 1998b) and adhered to Good Laboratory Practice Principles (OECD, 1998a). In the first experiment, human blood peripheral lymphocytes were exposed to 2'-FL at concentrations of up to 2,000 µg per mL in the presence and absence of metabolic activation for 3 hours with a 27-hour harvest time. In the second experiment, cells were exposed to 2'-FL for 24 hours with a 24-hour harvest time in the absence of S9 mix. Mitomycin C was the positive control in the absence of S9 and cyclophosphamide was the control in the absence of S9. Neither experiment showed evidence of precipitation or cytotoxicity. Doses of 510, 612, or 2,000 µg per mL did not result in a statistically significant increase in mononucleated or binucleated cells with micronuclei. There was no evidence of clastogenicity or aneugenicity in the presence of absence of S9 mix at up to 2,000 µg per mL, which was the highest concentration tested.

A preliminary *in vivo* micronucleus test examining rat bone marrow cells was conducted in which doses of 500, 1,000, or 2,000 mg per kg bw of Jennewein's 2'-FL were administered to one male and one female animal per dose. No systemic toxicity was observed; therefore, the same doses were used in the main study. In the main study, Jennewein's 2'-FL was administered by gavage to groups of 5 rats (Crl:CD(SD)) per sex (Jennewein, 2015) at doses of 500, 1,000, or 2,000 mg per kg bw per day. A vehicle control group received 0.8% aqueous hydroxypropylmethylcellulose and a positive control group was administered cyclophosphamide. The study was conducted in accordance with OECD Guideline 474. The rats were sacrificed at 24- or 48-hours post administration and bone marrow smears were evaluated by observing 2,000 erythrocytes per animal. There was no increase in the incidence of micronucleated polychromatic erythrocytes (PCEs) at any of the three tested dose levels of Jennewein's 2'-FL compared with the control. The positive control showed a significant increase in the number of micronuclei.

Glycosyn and FrieslandCampina agree that there was no evidence of induction of micronuclei *in vivo* in rats in any of the cell types examined by up to 2,000 µg per mL of Jennewein's 2'-FL. These studies are corroborative evidence that 2'-FL does not have any genotoxic activity and is not a carcinogenic risk to humans.

iii. Other Animal Studies

Vázquez et al. (2015) studied the effect of 2'-FL on hippocampal long term potentiation (LTP) and learning abilities in mice and rats. Chronic oral administration of 2'-FL resulted in mice and rats exhibited improved input/output curves and LTP in alert behaving animals. The improvement in LTP was associated with improved performance. Mice that were administered 2'-FL showed better performance than control animals in spatial learning, working memory, and operant conditioning as measured using the IntelliCage system. Similarly, improved performance was observed for rats

when tested in the fixed-ratio schedule in the Skinner box. Exposure to 2'-FL was associated with greater expression of molecules associated with storing newly acquired memories.

Vazquez et al. (2016) reported that addition of 0.625% (w/w) of 2'-FL but not 0.21% (w/w) of l-fucose to the diet of male Sprague Dawley adult rats improves operant conditioning and LTP associative learning related skills. These doses provided 350 mg of 2'-FL per kg bw per day. The authors stated this amount was similar to the dose found in breastmilk. The doses of 2'-FL and l-fucose were equimolar. The effect on 2'-FL on LTP was inhibited following vagotomy.

Oliveros et al. (2016) investigated whether oral supplementation with 2'-FL during the lactation period affects memory and learning in rats. Rat pups were orally administered 2'-FL or water during the lactation period and were then fed a standard diet and were assessed at weaning age 4-6 weeks and at age one year. At age 4 to 6 weeks, the behavior of rats in both groups was similar in the Morris Water Maze; however, the authors stated that the rats that were fed 2'-FL appeared to do slightly better in the test. When tested at one year of age, rats that were treated with 2'-FL exhibited better performance in the Novel Object Recognition and Y Maze paradigms compared with controls and had longer and more intense LTP in young and adult rats.

Castillo-Courtade et al. (2015) showed that following an oral ovalbumin challenge in sensitized 8-to 9-month-old male Balb/c mice, daily oral administration of 2'-FL or siallylactose reduced food allergy symptoms, such and diarrhea and hypothermia. In addition, there was a suppression of antigen-induced increases in mouse mast cell protease-1 in serum and mast cell numbers in the intestine, and increased CD4(+), CD25(+), and IL-10(+) in cells in the Peyer's patches and the mesenteric lymph nodes. Treatment with 6′-siallylactose was associated with elevated concentrations of IL-10 and reduced TNF production. While both HMOs reduced the passive cutaneous anaphylaxis response.

He et al. (2016) reported that 2'-FL reduces inflammation mediated by lipopolysaccharide in human enterocytes by decreasing the induction of CD14.

Glycosyn and FrieslandCampina conclude that the results of these studies do not raise any concerns about safety of 2'-FL.

5. Human Studies & Experience with 2'-FL Preparations

No clinical studies have been conducted on Glycosyn and FrieslandCampina's 2'-FL. Several published studies on 2'-FL from other sources are available and support the safety of 2'-FL in the human diet.

Marriage et al. (2015) conducted a prospective, randomized, controlled, growth and tolerance study in healthy, singleton infants with human breastmilk, a control formula containing 2.4 g per L GOS or formula containing 0.2 g per L of 2′-FL and 2.2 g per L of GOS, or 1.0 g per L 2′-FL and 1.4 g per L of GOS for 4 months. Study participants were enrolled in the study by age 5 days. The infant formulas contained 64.3 kcal per dL (19 kcal per fl. oz.) and had a similar caloric density as

breast milk. There was no significant difference in weight, length, or head circumference between intervention groups and the control group. There was no significant difference between groups in the percentage of study participants that experienced adverse events. The authors reported that the formulas were well tolerated and that there was comparable average stool consistency, number of stools per day, and percent of feedings associated with spitting up or vomiting.

The report on the safety of 2′-FL that was issued by the EFSA Panel on Dietetic Products, Nutrition, and Allergies (EFSA, 2015) reviewed a clinical study in infants that was submitted by Glycom and has been published since that time (Puccio et al., 2017). In this double-blind, randomized, controlled clinical trial, 175 healthy, full term infants ranging from 0 to 14 days of age were randomly assigned to be treated with formula containing a combination of 2′-FL and lacto-N-neotetraose (LNnT) (n=88, 1.0–1.2 grams per L of 2′-FL and 0.5–0.6 grams per L of LNnT for reconstituted formula) or formula that did not contain oligosaccharides (n=86) for up to 6 months. There was no inferiority of the weight gain of infants treated with 2'-FL and LNnT compared with those treated with the control formula until they were 4 months old. The mean weight, length, head circumference, and body mass index (BMI) for infants through age 4 months compared well with the WHO standard growth curves. The Panel was of the opinion that the data obtained on stool endpoints and the altered composition of the microbiota in infants that were given the oligosaccharide mixture gave no cause for concern about the safety of the mixture. Infants that were treated with 2'-FL and LNnT had significantly lower incidences of bronchitis that infants in the control group [odds ratio (OR) = 0.30; 95 % CI 0.11–0.73; p = 0.004]. Infants that were treated with 2'-FL and LNnT had significantly lower antibiotic use than infants in the control group (25.0 % vs. 41.4 %; OR = 0.47; 95 % CI 0.23–0.94, p = 0.025). There were no other significant differences in adverse effects between treatment and control groups. The dose of 2'-FL in infant formula in the study was approximately half of the maximum proposed level. The Panel noted that this concentration of 2′-FL would be equivalent to 1.27 grams per day and 209 mg per kg bw per day at the 95th percentile for an infant who is 3 months old and weighs 6 kg.

The opinion on the safety of 2′-FL that was issued by the EFSA Panel on Dietetic Products, Nutrition, and Allergies (EFSA, 2015) also described a placebo-controlled, double-blind, parallel, dose-response trial in 100 healthy adults (49 women and 51 men) that was submitted by Glycom and has since been published (Elison et al., 2016). Study participants were randomly assigned to one of 10 treatment groups (n=10 per group) in which they consumed single doses of 5, 10, or 20 grams per day of 2′-FL or LNnT alone; 5, 10, or 20 grams per day of a combination of 2′-FL and LNnT (with 2′-FL and LNnT in a 2:1 ratio), or glucose (placebo) each day for two weeks. In comparison with the placebo group, study participants who were treated with 20 grams per d of 2′-FL experienced increased incidences of nausea, rumbling, bloating, passing gas, diarrhea, loose stools, and urgency after two weeks. No significant increases in the incidences of these effects were reported in the 5 and 10 grams per day 2′-FL dose groups compared with the placebo group; however, for the 10 grams per day and 20 grams per day groups, an increased incidence of passing gas was noted. No significant differences in stool consistency were noted between placebo and intervention groups. Seventy-eight symptoms were reported by 44 study participants.

Gas/flatulence, stomach pain, and diarrhea and rumbling were reported most frequently; however, the adverse events were described as mild and no serious adverse events were noted.

In summary, the EFSA Panel for the evaluation of the safety of 2'-FL as a novel food ingredient (EFSA, 2015) concluded that 2'-FL:

- *"is safe for infants (up to one year of age) when added to infant and follow-on formulae, in combination with lacto-N-neotetraose (LNnT), at concentrations up to 1.2 g/L of 2'-FL and up to 0.6 g/L of LNnT, at a ratio of 2:1 in the reconstituted formulae;*
- *is safe for young children (older than one year of age) when added to follow-on and young-child formulae, at concentrations up to 1.2 g/L of 2'-FL (alone or in combination with LNnT, at concentrations up to 0.6 g/L, at a ratio of 2:1);*
- *is safe when added to other foods at the proposed uses and use levels."*

Goehring et al. (2016) conducted a substudy that was nested in a randomized controlled growth and tolerance trial to investigate the effect of supplementation of infant formula with 2'-FL on inflammatory cytokines. Healthy singleton infants were enrolled in the study starting on 5 days old. The infants were exclusively fed formula (n=317) or breastmilk (n=107) through age four months. Feeding infants formula to which 2'-FL had been added resulted in reduced plasma inflammatory cytokines profiles that resembled the inflammatory profiles of infants who were breastfed.

Sprenger et al. (2016) used logistic regression models to analyze the relationship between FUT dependent oligosaccharides in breastmilk and prevention and risk of allergies at ages 2 and 5 years using data from the placebo group in a randomized, placebo controlled study on prebiotics and probiotics and prevention of allergy. Infants who are delivered by C-section and who have a high hereditary risk of allergy potentially have a reduced risk of IgE associated eczema at age two years but not age 5 years when fed breastmilk containing FUT2-dependent oligosaccharide.

Sprenger et al. (2017) studied the relationships between the FUT2 status, concentration of major HMOs in breastmilk, and infant growth through 4 months of age in an open observatory, single center, longitudinal cohort study. Breastmilk was collected at 30, 60, and 120 days postpartum from 50 mothers, who gave birth to 25 female and 25 male singleton infants. 2'-FL decreased over time. Mothers were placed into two categories low 2'-FL, (mean 27 mg per L, 95% confidence interval of mean 12-42 mg per L) and high 2'-FL (mean 2,170 mg per L, 95% confidence interval of mean 1,880-2,460 mg per L). Individuals who had low concentrations of 2'-FL in their milk had lacto-N-tetraose (LNT) as the major HMO. The variation in HMOs for high and low clusters of 2'-FL has no effect on infants of either sex for body length, body weight, BMI, and head circumference.

Steenhout et al. (2016) (abstract) analyzed microbiota of stool samples from healthy infants at 3 months old who had been fed either a cow's milk-based infant formula (control, n=87) or the same formula with 1.0 g per L 2'-FL and 0.5 g per L LNnT (Test, n= 88) or were breastfed (reference group, n=38). They reported that supplementation of formula with 2'-FL and LNnT moves the microbiota and metabolic signature in stool closer to that of breastfed infants with respect to composition and function.

Lewis et al. (2015) compared the effects of feeding infant breastmilk from non-secretor mothers (milk lacking 2'-FL) with the effect of feeding infants breastmilk from secretor mothers (milk containing 2'-FL) on establishment of *Bifidobacteria*-rich microbiota on days 6, 21, 71, and 120 of life. Infants who were fed breastmilk from non-secretor mothers showed delayed establishment of *Bifidobacteria*-rich microbiota compared with infants who were fed breastmilk from secretor mothers.

Glycosyn and FrieslandCampina have reviewed these clinical studies and conclude that they support the safety of the proposed uses of 2'-FL for infants, toddlers and adults. In addition, the estimates that infants consuming breast milk may receive 600 mg per kg bw per day or more affirms the safety of the proposed use in infant formula where the highest exposure estimated is in consumer-only population group of infants aged 0 to 5 months is a mean of 315 mg per kg bw per day were estimated to have the highest mean and 90th percentile, of 315 mg per kg bw per day and 532 mg per kg bw per day, respectively. In addition, Glycosyn and FrieslandCampina conclude that the proposed use in medical foods for patients 11 years of age and older under the direction of a physician of a maximum of 4 grams per serving 3 times per day would be well tolerated and safe. We conclude that results of the clinical study by Elison et al. (2016) showing no serious GI disturbances at a single dose of 10 grams per person demonstrate that the proposed regimen for medical foods is safe and would be well tolerated.

6. Toxicology Studies on Substances that are Similar in Structure to 2'-FL

There is much interest in the nutritional benefit of oligosaccharides in the diet. As indicated in Table 16, there have been 20 oligosaccharide preparations that have been the subject of successful GRAS notifications to date. The safety evidence for these oligosaccharides at gram quantities in the human diet relies on similar studies; i.e. no evidence of toxicity in the highest level tested in subchronic rat studies; no evidence of genotoxicity in a variety of tests; and the absence of adverse events seen in clinical trials at proposed dietary levels. The available toxicology data for GOS, FOS, and an isomaltooligosaccharide (IMO) is briefly summarized below as examples of the general lack of toxicity of oligosaccharides.

a. Animal Toxicity Studies on Structurally Similar Substances

The most recent GRN for which GOS was the subject ingredient to receive a "no questions" letter from FDA (FDA, 2016a) was GRN 620. In their notification, Nestle Nutrition (2015) described repeated dose studies in which the no observed adverse effect level (NOAEL) was determined to be the highest dose given and ranged from 2,000 to 6,900 mg per kg bw per day (Anthony et al., 2006; Environ, 2007; Kobayash et al., 2014; Kobayashi et al., 2009; Penard, 2015b). Two reproductive/developmental toxicity studies were described in GRN 620. A one generation reproductive toxicity test reported a NOAEL for male and female parent animals and for the growth and development of their offspring of at least 2,000 mg per kg per day (Kobayashi et al., 2014). Desbuards et al. (2012) reported no toxicity in dams and pups that consumed a diet containing a

prebiotic consisting of galacto-oligosaccharides-inulin in a 9:1 ratio GOS, 1,620 mg per kg bw per day + inulin (400 mg per kg bw per day).

New Francisco Biotechnology Corporation (2016) was the most recent submitter of GRAS notification for FOS (GRN 623) to receive a "no questions" response from FDA (FDA, 2016b). The submitter noted that safety data on FOS has been reviewed by national and international regulatory agencies including FDA, EFSA (SCF) and FSANZ. In summary, an acute oral toxicity study in rats reported an LD_{50} of greater than 9,000 mg per kg bw per day of scFOS (Takeda and Niizato, 1982). Subacute oral toxicity studies reported NOAELs of 4,500 mg per kg bw per day (Takeda and Niizato, 1982) and a NOAEL was unreported by (Tokunaga et al., 1986).

In subchronic oral toxicity studies, NOAELs of oligofructose were 4,680 and 5,720 mg per kg bw per day in male and female rats (Boyle et al., 2008). In an oral toxicity study in which Wistar rats were fed yacon flour containing oligofructose, the NOAEL was determined to be 6,800 mg per kg bw per day, equivalent to 3,760 mg oligofructose per kg bw per day (Genta et al., 2005). A two-year chronic toxicity study and carcinogenicity study reported that scFOS was not toxic or carcinogenic at dose of 0, 0.8, 2.0%, and 5.0% dietary concentration in Fischer 344 rats, equivalent to 0, 341, 854, and 2,170 in male rats and 0, 419, 1,045, and 2,664 mg per kg bw per day in female rats (Clevenger et al., 1988). Two unpublished developmental toxicity studies were reported on oligofructose. One showed that oligofructose at a dietary concentration of 20% had no effect on pregnancy or on the development of rats (Henquin, 1988). Another developmental toxicity study showed that when oligofructose was administered at 4.75% of the diet from post coitum day 0 to 6, and then at 0, 5, 10, or 20% of the diet, there were no adverse effects and did not adversely affect pregnancy outcomes or in utero or development of rats (Sleet and Brightwell, 1990).

In 2017, GRN 674 received a "no questions" response from FDA for isomaltooligosaccharide (IMO) (FDA, 2017a). As part of its discussion on safety, GRN 674 described the results of a repeat-dose study in what was described as a study article, which was written in Japanese with a short summary that was written in English (Day and Chung, 2007). The authors of GRN 674 also noted that the study design did not appear to adhere to established guidelines for short term studies, such as OECD guidelines (BioNeutra North America, 2017). In summary, male Sprague Dawley rats were administered an IMO mixture comprised of saccharides with a degree of polymerization of 3 or greater (VF-DP3-IMO) at 20% of the diet (approximately 20 g per kg bw per day) for 6 weeks. There were no effects on body weight or food intake; however, there was a dose-dependent decrease in abdominal body fat. A significant increase in the weight of the cecum was observed, which was proposed as being associated with an increase in the colonic bacterial population. The authors of GRN 674 stated that this study provided evidence that IMOs are not a concern as relates to the risk of systemic toxicity.

b. Genotoxicity and Mutagenicity Studies on Structurally Similar Substances

With respect to substances that are structurally similar to 2'-FL, comprehensive evaluations demonstrating the safety of the structurally similar GOS included bacterial reverse mutation assays

and *in vitro* chromosomal aberration tests, as well as *in vivo* micronucleus assays in mice following administration of GOS by gavage and the Comet assay (Kobayashi et al., 2009; Narumi et al., 2014; Verbaan, 2015c; Verspeek-Rip, 2015b; Yasutake et al., 2003). GOS was not mutagenic and did not induce chromosomal aberrations *in vitro*, nor did it induce micronuclei *in vivo* at up to the highest doses tested. The synthetic HMO, LNnT, was also not genotoxic (Coulet et al., 2013).

In addition, comprehensive evaluations demonstrating the safety of the structurally similar GOS (GTC Nutrition, 2009a; b; Yakult, 2010) included clinical studies in healthy and compromised adults and preterm and term infants.

7. Allergenicity

No reports of allergenicity of 2'-FL were found in the published literature. There are no reports of allergenicity to the proteins from the fermentation organism. In addition, all batches will meet the maximum residual protein specification of 0.01% making any allergic hazard unlikely. In an opinion on the safety of 2'-FL as a novel food ingredient, the EFSA Panel on Dietetic Products, Nutrition and Allergies stated that since no detectable proteins or peptides were present in Glycom's manufactured 2'-FL, no allergenicity of 2'-FL was expected (EFSA, 2015).

Lactose is one of the raw materials used to produce 2'-FL. As also described in GRN 650, in order to comply with the Food Allergen Labeling and Consumer Protection Act (FALCPA), the bulk ingredient would have to be labelled "contains milk" or "contains milk ingredients."

C. Expert Panel Findings on Safety of Purified 2'-Fucosyllactose (2'-FL)

At the request of Glycosyn, LLC and FrieslandCampina, GRAS Associates convened an expert panel to independently evaluate the available information in order to determine whether Glycosyn and FrieslandCampina's purified 2'-FL (purity >90%) can be considered GRAS when used as an ingredient in infant formulas, conventional foods, and medical foods. Uses in medical foods would be under the direction of a physician. The expert panel has reviewed the chemistry of Glycosyn and FrieslandCampina's 2'-FL, the process for producing 2'-FL, and all available published and unpublished safety data in its evaluation of the GRAS status of 2'-FL.

As a simple trisaccharide of L-fucose, d-galactose, and D-glucose, there is a high presumption that 2'-FL is safe for human consumption. The Expert Panel notes that the scientific literature establishes that as with other human milk oligosaccharides that 2'-FL is partially absorbed from the GI tract. Unabsorbed 2'-FL is partially fermented by intestinal biota and exerts a prebiotic effect that promotes intestinal homeostasis.

It is noted that FDA has issued "no questions" letters (U.S. FDA, 2015a; b; 2016) in response to three previous GRAS notices on 2'-FL produced by different manufacturing processes as described in GRNs 546, 571 and 650 (Glycom, 2014; 2016; Jennewein, 2015). In addition, the European Food Safety Authority (EFSA) conducted a safety evaluation of 2'-FL at the request of Glycom and determined that 2'-FL was safe when used as proposed (EFSA, 2015). The Expert

Panel finds that the purity specifications for Glycosyn and FrieslandCampina's 2'-FL are adequate and comparable to previously notified products.

The Expert Panel has carefully reviewed the manufacturing process, including the nature of the bioengineered organism used in the fermentation process. The background organism upon which all genetic manipulations were performed is the non-pathogenic bacterium *E. coli* K12, the same host organism as is widely used to produce many oral and injectable human drugs. A related non-pathogenic *E. coli* strain (*E. coli* "Nissle") has been used safely as a probiotic for 100 years. There are no virulence genes in *E. coli* K12, and in particular the specific strain of *E. coli* K12 used by Glycosyn and FrieslandCampina (ATCC 55151; *E. coli* K12 GI724), as confirmed by whole genome sequencing. All genetic modifications are well documented in filed patents and in the dossier. Extensive purification steps remove viable cells, cell debris, and protein and peptide particles. Extensive qPCR testing demonstrated that no residual DNA from the genetically modified *E. coli* production strain was present in the final 2'-FL product. Final purity specifications established for the final 2'-FL product are in close agreement with established specifications for 2'-FL products presented in multiple GRNs that received "no questions" letters from FDA.

There are adequate published toxicology studies in the scientific literature for 2'-FL. A 14-day oral tolerability study in rats using doses of 2, 5, and 7.5 g per kg bw per day was used to set a high dose of 6,000 mg per kg bw per day for a 90-day oral toxicity study (Coulet et al., 2014). There were no adverse effects attributed to 2'-FL seen in a 90-day rat study using neonatal rats (from postnatal day 7 through postnatal day 98) at doses of up to 5 g per kg bw per day (Coulet et al., 2014). The EFSA Panel on Dietetic Products, Nutrition, and Allergies reviewed the 90-day subchronic toxicity conducted by Coulet et al. (2014) as described in Glycom's submission for consideration of 2'-FL as a new food ingredient and took a more conservative approach to the NOAEL, concluding that the NOAEL was 2,000 mg per kg bw per day (EFSA, 2015) on the basis of a reduction in the relative kidney weight for females in the 6,000 mg per kg bw per day dose group, two unexplained deaths of animals in the 6,000 mg per kg bw per day dose group, and some significant differences observed in the hematological and clinical chemistry parameters in the 5,000 and 6,000 mg per kg bw per day dose groups (EFSA, 2015). In GRNs 571 and 650, which both received "no questions" responses from FDA, the expert panels agreed with Coulet et al. (2014) that the NOAEL was 5,000 mg per kg bw per day (Glycom, 2016). The Expert Panel agrees with Glycom that the NOAEL for this study is 5,000 mg per kg bw per day.

In another published study, no adverse effects on growth and development, clinical pathology, or histopathology were seen in piglets fed a liquid diet to which of 200, 500, or 2,000 mg per L of Jennewein's 2'-FL starting was added starting at postnatal day 2 for 3-weeks (Hanlon and Thorsrud, 2014). These doses were equivalent to 29.37, 72.22, and 291.74 mg per kg bw per day in male piglets and 29.30, 74.31 and 298.99 mg per kg bw per day of 2'-FL in female rats.

Coulet et al. (2014) also reported that no mutagenic activity of 2'-FL was seen in a bacterial mutagenicity test and no clastogenic or aneuploidic effect was seen in a mouse lymphoma assay.

The designs of these studies support the proposed uses using young animals, and not only supports the safe use of 2'-FL for human adults but also for infants, toddlers and children.

The Expert Panel has compared the chemical composition and specifications of Glycosyn and FrieslandCampina's 2'-FL to the test materials used in these published studies described in previous GRNs and has found Glycosyn and FrieslandCampina's 2'-FL to be substantially equivalent. As a result, the Expert Panel has concluded that these studies support the safety of the Glycosyn and FrieslandCampina product.

Corroborative evidence of the safety of 2'-FL is obtained in unpublished studies on Glycosyn and FrieslandCampina's 2'-FL. FrieslandCampina has conducted confirmatory toxicology studies on the subject 2'-FL at Triskelion Laboratories. No adverse effects were seen in a 90-day rat study where the diets contained 2'-FL at 3, 6, and 10% of the diet. In this study, an increase in cecal weights was seen at the mid- and high- dose that was previously not reported in other rat studies. The investigators concluded that this is a common finding in studies on poorly digestible carbohydrates including dietary fiber. There were no adverse histopathological findings in the cecum in the high dose group. The Expert Panel notes that the cecum is not normally weighed in standard toxicology protocols and concludes that this effect may have been overlooked by previous investigators. The Expert Panel agrees that the effects in the cecum are adaptive based on the nature of the test material and that the NOAEL for this study was the highest dose tested, which is equivalent to 7.25 and 7.76 g per kg bw per day, for males and females, respectively.

Other unpublished *in vitro* toxicology studies provide evidence of the safety of 2'-FL. FrieslandCampina conducted a study that showed no mutagenicity of Glycosyn and FrieslandCampina's 2'-FL at doses of up to 5,000 µg per plate in the absence or presence of metabolic activation, and no clastogenic or aneugenic activity was noted when Glycosyn and FrieslandCampina's 2'-FL was added at doses of up to 2,000 µg per mL in cultured binucleated human lymphocytes. The safety of 2'-FL was also shown in other unpublished studies.

No clinical studies have been conducted on Glycosyn and FrieslandCampina's 2'-FL; however, three published clinical studies provided evidence of the safety of 2'-FL. In a clinical study in which infants were fed formula containing a combination of 2'-FL and lacto-N-neotetraose (LNnT) (n=88, 1.0–1.2 grams per L of 2'-FL and 0.5–0.6 grams per L of LNnT for reconstituted formula) or formula that did not contain oligosaccharides (n=86) for up to 4 months, it was found that the formula was safe and well tolerated and supported age appropriate growth (Puccio et al., 2017). Elison et al. (2016) conducted a study in which adults were fed 5, 10, or 20 g per day of 2'-FL or LNnT alone, 5, 10, or 20 g per day of a combination of 2'-FL and LNnT (2:1), or glucose (placebo) for 2 weeks. Increased gastrointestinal effects were observed in to 20 g per day dose groups, but there were no significant differences in stool consistency and adverse events were mild. The Puccio et al. (2017) and Elison et al. (2016) studies were reviewed by the EFSA Panel on Dietetic Products, Nutrition and Allergies as a part of their safety evaluation of 2'-FL in 2015 (EFSA, 2015).

A third study investigated the effects of two combinations of 2'-FL when added to infant formula in combination with GOS (0.2 g per L of 2'-FL and 2.2 g per L of GOS, or 1 g per L of 2'-FL and 1.4 g per L of GOS). There were no significant differences between these treatment groups and the control group (2.4 g per L of GOS) in terms of the number of adverse events, growth from age 5 days to 4 months, and tolerance (Marriage et al., 2015). The formulas were described by the authors as being well tolerated.

The mean and 90th percentile consumer-only intakes of 2'-FL in the total population (consumers of all ages) were determined to be 1.70 g per person per day and 3.54 g per person per day, respectively. Infants aged 6 to 11 months were determined to have the highest mean consumer-only intakes, at 2.28 g 2'-FL per person per day, while male teenagers were determined to have the highest 90th percentile intake, at 4.29 g 2'-FL per person per day. The lowest estimated mean and 90th percentile EDIs for 2'-FL were determined for females of childbearing age (aged 16 to 45 years), at 1.36 g per person per day and 2.87 g per person per day, respectively.

Consumer-only intakes of 2'-FL in the total population were also determined on a body weight basis, where the mean and 90th percentile estimates were determined to be 36 mg per kg bw per day and 80 mg per kg bw per day, respectively. Of all consumer-only population groups, infants aged 0 to 5 months were estimated to have the highest mean and 90th percentile, of 315 mg per kg bw per day and 532 mg per kg bw per day, respectively. This compares favorably to estimates of maximum exposure to 2'-FL of 600 mg per kg bw or more for breastfed infants. The lowest mean and 90th percentile EDIs for 2'-FL were determined for adult females and females of childbearing age, at 20 mg per kg bw per day and 43 mg per kg bw per day, respectively.

In addition, Glycosyn and FrieslandCampina propose use in medical foods at a level of 12 g per person per day (200 mg per kg bw for a 60 kg adult). This would be the sole source of 2'-FL in the diets of these patients as they are not expected to consume conventional foods with 2'-FL.

In comparing the NOAEL on 5,000 mg per kg bw per day in the published subchronic rat study (Coulet et al., 2014) to proposed use levels, the Expert Panel notes that the level of 6,000 mg kg bw per day was not considered the NOAEL because of the unexplained death of two animals at this dose. The Expert Panel notes that deaths were not seen in the highest dose tested in the unpublished FrieslandCampina and Jennewein studies; these doses were 7.25-7.76 g per kg bw per day and 7.76-8.72 per kg bw per day, respectively.

Using the most conservative approach, the calculated margin of safety from the consumer-only intakes of 2'-FL in the total population to the mean of 36 mg per kg bw per day and 90th percentile level of 80 mg per kg bw per day to the NOAEL of 5,000 mg per kg bw per day is 138- and 62-fold, respectively.

Of all consumer-only population groups, infants aged 0 to 5 months were estimated to have the highest mean and 90th percentile, of 315 mg per kg bw per day and 532 mg per kg bw per day, respectively. Infants would consume high levels only for the time they consumed formula. These

levels have been shown to be safe in the published clinical studies on infants and are similar to levels noted to be consumed by breast fed infants.

The highest use expected from use in medical foods is 12 g per person per day or 200 mg per kg bw per day. The conservative margin of safety from the NOAEL of 5,000 mg per kg for the use in medical foods is 25-fold. The expert panel also notes that they believe that the published clinical study (Elison et al., 2016) in adults with doses of 5, 10, and 20 g per day supports the safety of the use in medical foods. While mild GI disturbances were seen at 20g per day, the Panel notes that the dose in this study was given as a single dose. The Panel believes that the planned use of 4 g 2'-FL per serving given 3 times per day in medical foods will be well tolerated. The Expert Panel also notes that the medical food use would be supervised by a physician.

Based on this review of all the safety evidence, the Expert Panel[6] concludes that the proposed uses of 2'-FL in infant formulas and conventional foods for toddlers, children and adults, and in medical foods are safe.

(b) (6)

Richard Kraska, Ph.D., DABT

Chair

(b) (6)

Doug Archer, Ph.D.

(b) (6)

Katrina V. Emmel, Ph.D.

D. Common Knowledge Elements for GRAS Conclusions

The first common knowledge element for a GRAS conclusion requires that data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing studies published in peer-reviewed scientific journals. The second common knowledge

[6] Dr. Kraska worked on GRAS and food additive safety issues within FDA's GRAS Review Branch earlier in his career, and subsequently continued working within this area in the private sector. Drs. Archer and Emmel have substantial food safety experience. All three panelists have extensive technical backgrounds in the evaluation of food ingredient safety and in participating in the deliberations of GRAS Expert Panels. Dr. Kraska served as Chair of the Panel.

element for a GRAS conclusion requires that there must be a basis to conclude that consensus exists among qualified scientists about the safety of the substance for its intended use.

1. Public Availability of Scientific Information

There is much published scientific evidence that 2'- FL is a common constituent of human milk. There are ample safety studies in the published scientific literature to support the safety of added 2'-FL in the diet of infants, children, and adults. The principal toxicology studies include a 90-day study in rats as well as genotoxicity studies (Coulet et al., 2014) and a study in weanling pigs (Hanlon and Thorsrud, 2014). Additional corroborative support for the safety of 2'-FL also comes from several unpublished *in vitro* and animal toxicity studies including studies conducted by FrieslandCampina. FrieslandCampina is in the process of preparing manuscripts for publication of the studies in peer reviewed scientific journals. These publications will enhance the public availability of scientific information supporting the safety of 2'-FL.

The primary publicly available clinical studies reviewed include two that were conducted in infants (Marriage et al., 2015; Puccio et al., 2017) and one conducted in adults (Elison et al., 2016). These studies support the safe use of 2'-FL.

There is good scientific evidence that the donor and recipient organisms used to engineer the fermentation organism is safe.

2. Scientific Consensus

FDA has reviewed three previous GRNs related to 2'-FL (Glycom, 2014; 2016; Jennewein, 2015) and responded with "no questions." In addition, the EFSA Panel on Dietetic Products, Nutrition and Allergies concluded that 2'-FL "is safe for infants (up to one year of age) when added to infant and follow-on formulae, in combination with lacto-N-neotetraose (LNnT), at concentrations up to 1.2 g per L of 2'-FL and up to 0.6 g per L of LNnT, at a ratio of 2:1 in the reconstituted formulae; is safe for young children (older than one year of age) when added to follow-on and young-child formulae, at concentrations up to 1.2 g per L of 2'-FL (alone or in combination with LNnT, at concentrations up to 0.6 g per L, at a ratio of 2:1); is safe when added to other foods at the proposed uses and use levels" (EFSA, 2015).

E. Conclusion

In consideration of the aggregate safety information available on 2'-FL, Glycosyn, FrieslandCampina, and the designated Expert Panel conclude that the 2'-FL defined in the subject notification and produced under Good Manufacturing Practices is safe for use in term infant formulas, conventional foods and medical foods as described within this GRAS notification, and is generally recognized as safe (GRAS) within the meaning of the Food, Drug, and Cosmetic Act.

This declaration has been made in accordance with FDA's standard for food ingredient safety, i.e., reasonable certainty of no harm under the intended conditions of use.

PART 7. LIST OF SUPPORTING DATA AND INFORMATION IN THE GRAS NOTICE.

A. List of Acronyms

ADI	Acceptable Daily Intake
ATCC	American Type Culture Collection
BMI	Body Mass Index
bp	base pairs
bw	body weight
B.V.	Besloten Vennootschap (Ltd., private company)
CASRN	Chemical Abstract Service Registry Number
CAS	Chemical Abstract Service
CCRC	Complex Carbohydrate Research Center
CFR	Code of Federal Regulations
CFU	Colony Forming Unit
DF	Dam-fed
dL	deciliter
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DSS	Dextran sulfate sodium
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
EGF	Epidermal Growth Factor
eNOS	endothelial nitric oxide synthase
ETEC	Enterotoxigenic *Escherichia coli*
EU	European Union

EU/mg	Endotoxin Unit per milligram
FAO	Food and Agriculture Organization of the United Nations
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FD&C	Federal Food, Drug, and Cosmetic Act
FF	Formula-fed
2'-FL	2'-Fucosyllactose
3-FL	3-fucosyllactose
fl. oz.	fluid ounce
FNDDS	Food and Nutrition Database for Dietary Studies
FOIA	Freedom of Information Act
FOS	Fructooligosaccharides
FucT	Fucosyltransferase
FutN	α1,2 fucosyltransferase
g	gram
GA	GRAS Associates
Gal	Galactose
G-CSF	Granulocyte-colony stimulating factor
GDP-L-fucose	Guanosine 5'-diphospho-β-L-fucose
GI	Gastrointestinal
Glc	Glucose
GMP	Good Manufacturing Process
GOS	Galacto-oligosaccharides
GRAS	Generally Recognized as Safe

GRN	GRAS Notification
HMO(s)	Human milk oligosaccharides
HPAEC-PAD	High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
HPLC	High-Performance Liquid Chromatography
IMO	Isomaltooligosaccharide
JECFA	Joint FAO/WHO Expert Committee on Food Additives
kcal	kilocalorie
kg	kilogram
L	Liter
LD_{50}	Lethal Dose, 50%
LDFT	Lactodifucotetraose
LLC	Limited Liability Corporation
LNnT	lacto-N-neotetraose
LPS	Lipopolysaccharide
LTP	Long term potentiation
max.	maximum
MCB	Master Cell Bank
MF	Microfiltration
mg	milligram
min.	minimum
mL	milliliter
n	number
NA	Not available

NAPRALETSM	Natural Product Alert
NCHS	National Center for Health Statistics
NEC	Necrotizing enterocolitis
NF	The National Formulary
NHANES	National Health and Nutrition Examination Surveys
NMR	Nuclear Magnetic Resonance
NOAEL	No Observed Adverse Effect Level
NS	Not Specified
OD	Optical density
OECD	Organisation for Economic Co-operation and Development
OF	Oligofructose
OR	Odds Ratio
PCE	Polychromatic erythrocytes
PETG	Polyethylene terephthalate glycol-modified
Ph. Eur.	European Pharmacopeia
PND	Post-natal day
ppm	Parts per million
qPCR	Quantitative Polymerase Chain Reaction
RACC	Reference Amounts Customarily Consumed
RO	Reverse osmosis
rpm	Revolutions per minute
RT	Retention Time
RTECS	Registry of Toxic Effects of Chemical Substances
thyA	Thymidylate synthase

TNFα	Tumor Necrosis Factor alpha
TOXNET	Toxicology Data Network
t-PA	Tissue plasminogen activator
UDP-glucose	Uridine diphosphate glucose
μg	microgram
μL	microliter
μM	micromolar
US	United States
USP	United States Pharmacopeia
WHO	World Health Organization
WCB	Working Cell Bank

B. References

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