ORIGINAL SUBMISSION

Glycom A/S, Diplomvej 373 Ø, 1st fl., DK - 2800 Kongens Lyngby

#650

GLYCOM

GRN 000650

April 21, 2016

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Dear Dr. Gaynor:

Re: GRAS Exemption Claim for 2'-*O*-fucosyllactose

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized as Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting one hard copy and one electronic copy (on CD), as the notifier [Glycom A/S, Diplomvej 373, DK-2800 Kgs. Lyngby, Denmark], a Notice of the determination, on the basis of scientific procedures, that 2'-*O*-fucosyllactose produced by microbial fermentation by Glycom A/S, as defined in the enclosed documents, is GRAS under specific conditions of use in term infant formula and in food, and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act*. This ingredient is chemically equivalent to the synthetic 2'-*O*-fucosyllactose notified to the FDA on October 20, 2014 (designated as GRN 546) and is intended for use as an alternative to the existing GRAS uses described therein. Information setting forth the basis for the GRAS determination, which includes detailed information on the notified substance and a summary of the basis for the GRAS determination, as well as a consensus opinion of an independent panel of experts in support of the safety of 2'-*O*-fucosyllactose under the intended conditions of use, also are enclosed for review by the agency.

I certify that the enclosed electronic files were scanned for viruses prior to submission and are thus certified as being virus-free using Symantec Endpoint Protection Virus and Spyware Protection (Definition April 20-16 r5).

Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner.

Sincerely,

(b) (6)

Christoph H. Röhrig, Ph.D.
Senior Scientist, Regulatory Affairs Manager
Glycom A/S

Glycom A/S, Diplomvej 373 Ø, 1st fl., DK - 2800 Kongens Lyngby

April 21, 2016

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Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
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5100 Paint Branch Parkway
College Park, MD 20740-3835

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Christoph H. Röhrig, Ph.D.
Senior Scientist, Regulatory Affairs Manager
Glycom A/S

GRAS Exemption Claim for 2'-*O*-Fucosyllactose (2'-FL) Produced by Fermentation

Submitted to:

Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835
USA

Submitted by:

Glycom A/S
Diplomvej 373
DK-2800 Kgs. Lyngby
Denmark

April 19, 2016

GRAS Exemption Claim for 2'-*O*-Fucosyllactose (2'-FL)

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I. GRAS EXEMPTION CLAIM

I.A Claim of Exemption From the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997)]

Glycom A/S hereby claims that the use of 2'-*O*-fucosyllactose (2'-FL) in term infant formula and conventional food and beverage products, as described in Section I.D below, is exempt from the requirement of premarket approval of the *Federal Food, Drug, and Cosmetic Act* because we have determined that such uses are Generally Recognized as Safe (GRAS).

Signed,

(b) (6)

20 April 2016

Christoph H. Röhrig, Ph.D.
Senior Scientist & Regulatory Affairs Manager
Glycom A/S
christoph.roehrig@glycom.com

Date

I.B Name and Address of Notifier

Glycom A/S
Diplomvej 373
DK-2800 Kgs. Lyngby
Denmark
Tel: +45 4525 2247
Fax: +45 3841 1720

I.C Common Name of the Notified Substance

2'-*O*-Fucosyllactose

I.D Conditions of Intended Use

Infant Formula

2'-*O*-Fucosyllactose is intended for use in term non-exempt infant formulas at a use level of up to 2,400 mg/L of the ready-to-drink or reconstituted formula. The proposed use level of 2'-FL in term non-exempt infant formulas is based on providing dietary intakes of 2'-FL that are representative of those occurring among infants fed mature human breast milk, which is reported to contain average concentrations of 2'-FL in the range of 1,100 to 4,260 mg/L. Higher concentrations of up to 7,000 mg/L also have been reported (see Section IV.A).

Conventional Food and Beverage Products

2'-*O*-Fucosyllactose also is intended for use in a variety of conventional food and beverage products across multiple categories as described in Table I.D-1.

Table I.D-1 Summary of the Individual Proposed Uses and Use Levels for 2'-FL in Conventional Food and Beverage Products and Infant Formula

Food Category	Proposed Food-Uses	RACC[a]	Proposed Maximum Use Level (g/RACC)	Proposed Maximum Use Level (g/kg or g/L)[b]
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	1.2	5
	Sports, Isotonic, and Energy Drinks	240 mL	0.28	1.2
Dairy Product Analogs	Imitation Milks	240 mL	0.28	1.2
	Non-Dairy Yogurt	225 g	1.2	5.3
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.24	2.4
	Toddler Formulas	100 mL[c]	0.24	2.4
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	30 g	1.2	40
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk[d]	240 mL	0.28	1.2
Milk Products	Buttermilk	240 mL	0.28	1.2
	Flavored Milk	240 mL	0.28	1.2
	Milk-Based Meal Replacement Beverages, for Weight Reduction	240 mL	1.2	5
	Yogurt	225 g	1.2	5.3
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.28	1.2

2'-FL = 2'-*O*-fucosyllactose; RACC = Reference Amounts Customarily Consumed; U.S. = United States.
[a] Serving sizes were based on RACCs per Eating Occasion in the United States Code of Federal Regulations (21 CFR §101.12 - U.S. FDA, 2015a).
[b] The proposed maximum use level is presented on a g/kg basis for solids and on a g/L basis for liquids.
[c] RACC not available, 100 mL employed as an approximation.
[d] Milk is a standardized food in the United States. When the milk is fortified with 2'-FL, it will then be classified as a milk product.

I.E Basis for the GRAS Determination

Pursuant to 21 CFR § 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2015b), 2'-*O*-fucosyllactose produced by microbial fermentation has been determined by Glycom A/S to be GRAS for uses in non-exempt term infant formula and specified conventional food and beverage products, as described herein, on the basis of scientific procedures.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the United States (U.S.) Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

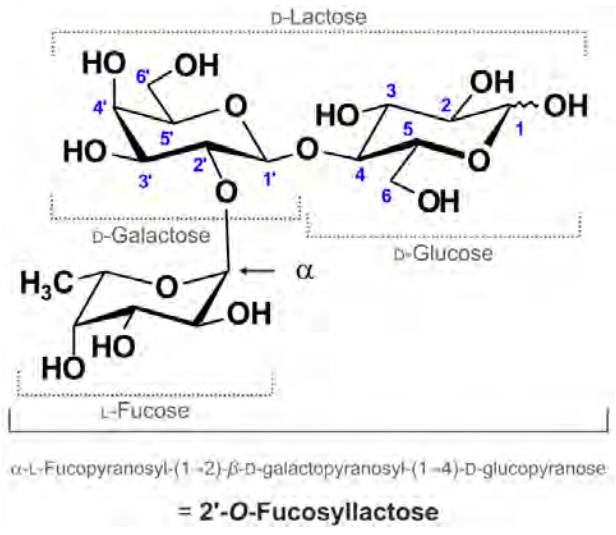
Glycom A/S
Diplomvej 373
DK-2800 Kgs. Lyngby
Denmark

Should the FDA have any questions or additional information requests regarding this notification, Glycom will supply these data and information.

II. DETAILED INFORMATION ABOUT THE IDENTITY OF THE SUBSTANCE

II.A Identity

II.A.1 Chemical Identity

Common Name:	2'-*O*-Fucosyllactose
Common Abbreviation:	2'-FL (2'FL, 2-FL, 2FL)
IUPAC Name:	α-D-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Alternative Denotations:	2'-*O*-L-Fucosyl-D-lactose; Fucosyl-α-1,2-galactosyl-β-1,4-glucose; Fuc-α-(1→2)-Gal-β-(1→4)-Glc
Chemical Abstracts Service (CAS) Registry Number:	41263-94-9
Chemical Formula:	$C_{18}H_{32}O_{15}$
Molecular Weight:	488.44
Structural Formula:	

II.A.2 Chemical and Physical Characteristics

2'-FL is a trisaccharide consisting of L-fucose, D-galactose, and D-glucose. Alternatively, 2'-FL can be described as consisting of the monosaccharide L-fucose and the disaccharide D-lactose, which are linked by an alpha(1→2) bond to form the trisaccharide. The molecular structure of 2'-FL was first elucidated by Richard Kuhn in 1955 (using classical chemical techniques) (Kuhn *et al.*, 1955) and shortly thereafter by Jean Montreuil (Montreuil, 1956). Since then the structure of 2'-FL was confirmed independently by others using a range of modern structure characterization techniques, including spectroscopic techniques [*e.g.*, $_{1}$H-, $_{13}$C, and 2D-nuclear magnetic resonance (NMR)] (Jenkins *et al.*, 1984; Ishizuka *et al.*, 1999; Rundlöf and Widmalm, 2001; Urashima *et al.*, 2002, 2004, 2005; Almond *et al.*, 2004; Wada *et al.*, 2008), mass spectrometric (MS) techniques (Fura and Leary, 1993; Asres and Perreault, 1996; Perreault and Costello, 1999), and x-ray crystallography (Kuhn *et al*., 1956; Svensson *et al*., 2002). 2'-FL is a naturally occurring trisaccharide found in mammalian milk with the highest concentrations occurring in *human* milk, and is therefore typically referred to as a *human* milk oligosaccharide (HMO). An oligosaccharide is a generic term for a saccharide polymer with a typical degree of polymerization below 20 (~3-20). Whilst human milk contains a combination of oligosaccharides (HMOs) it is important to emphasize that 2'-FL is a clearly defined trisaccharide that occurs only as one specific constitutional isomer. In simple terms, it is as clearly defined, for example, as sucrose is as a disaccharide consisting of glucose and fructose.

2'-FL produced by fermentation with *Escherichia coli* (*E. coli*) K-12 SCR6 is chemically and structurally identical to the 2'-FL produced from Glycom's chemical synthesis methods as described in GRN 546, and to 2'-FL that is present in human breast milk, as confirmed by ^{1}H- and 2D-nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry and x-ray crystallography.

II.B Method of Manufacture

2'-FL described herein is manufactured in compliance with cGMP and Glycom utilizes quality management systems based on the principles of Hazard Analysis Critical Control Point (HACCP) standards. The manufacturing process can be broadly divided into 2 stages. In Stage 1 [upstream processing (USP)], D-lactose and D-sucrose are converted to 2'-FL by the cellular enzymes of the 2'-FL production microorganism, which uses D-sucrose as an exclusive energy and carbon source and D-lactose as a substrate for 2'-FL biosynthesis. In Stage 2 [downstream processing (DSP)], a series of purification and isolation steps are used to generate the final high-purity 2'-FL ingredient.

II.B.1 Production Microorganism

II.B.1.1 Host

The genotypic characteristics of the host organism, *E. coli* K-12 DH1, are presented in Table II.B.1.1-1 below. The genome of *E. coli* K-12 has been sequenced and bioinformatic comparisons of the genome of *E. coli* K-12 with other safe laboratory strains and various pathogenic isolates have been conducted (Blattner *et al.*, 1997; Lukjancenko *et al.*, 2010). *E. coli* K-12 DH1 (*λ⁻ gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44*) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) culture collection, and the construction of strain DH1 has been described in the literature (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996).

Table II.B.1.1-1 Characteristics of the Host Organism *Escherichia coli* K-12 DH1

Genotype	*F⁻*, *λ⁻*, *gyrA96, recA1*, *relA1*, *endA1*, *thi-1*, *hsdR17*, *supE44*.
Genus	*Escherichia*
Species	*Escherichia coli*
Subspecies	not applicable
Strain	*E. coli* strain K-12 DH1
Culture collection	The German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen)
Deposition number	DSM 4235 (ATCC33849)

The DH1 strain is resistant to nalidixic acid due to the gyrA96 mutation (Hanahan, 1983). The K-12-derived strains cannot colonize the human gastrointestinal system, and do not produce protein-type toxins (U.S. EPA, 1997). The strain can grow in minimal medium, provided that it is supplemented with thiamine due to the thi-1 mutation. Further, the recA1 mutation minimizes the recombination and increases the stability of plasmids and chromosomal DNA of the strain.

II.B.1.2 Host Modification

The host strain *E. coli* K-12 DH1 (DSMZ, 2015) was optimized for general oligosaccharide expression features (used as a “platform host strain”) by the introduction of 7 modification events related to the metabolism of various sugars, thereby improving the efficiency of the strain, which was then called MDO. The 7 modifications that lead from DH1 to MDO are described below:

1. *lacZ* knockout

The *lacZ* gene encodes a β-galactosidase that hydrolyses lactose. The knockout of the *lacZ* gene was performed to prevent the hydrolysis of lactose which is used as a precursor for the synthesis of 2’-*O*-fucosyllactose. As a result of this knockout, the strain cannot grow on lactose. To construct the *lacZ* defective strain DHZ from strain DH1, a 1.545 kb DNA segment of the

lacZ gene, was deleted using the method of Hamilton *et al*. (Hamilton *et al.*, 1989). The construction of the suicide plasmid pMAK705 carrying the truncated *lacZ* sequence is described in detail in Dumon *et al.* (Dumon *et al.*, 2004). After the deletion the strain was called DHZ.

2. Insertion of Plac promoter

The introduced genes required for the synthesis of GDP-fucose (the sugar donor used in biochemical fucosylation of lactose to produce 2'-*O*-fucosyllactose) are part of the colanic acid gene cluster and are only induced in conditions of physiological stress during which the bacteria produce the extracellular polysaccharide colanic acid. To ensure a GDP-fucose production under normal physiological conditions, a Plac promoter has been inserted upstream of the *gmd* gene allowing induction of the GDP-fucose synthesis by the addition of the galactose derivative isopropyl β-D-1-thiogalactopyranoside (IPTG). This genomic modification does not modify the phenotype of the strain. The modified strain is designated strain ZL.

3. *nanKETA* knockout

Strain MDO is a general "platform host strain" used as starting point to generate derived strains for the production of a large diversity of human milk oligosaccharides and other bioactive oligosaccharides. A large fraction of these oligosaccharides are sialylated and their bacterial synthesis requires the knockout of the *nanK* and *nanA* gene to prevent the catabolism of precursors (sialic acid and N-acetylmannosamine) of CMP-Neu5Ac which is the sugar donor used in enzymatic sialylation (Fierfort and Samain, 2008). As a result of this knockout, the strain cannot grow on sialic acid. To construct strain ZLK from strain ZL, the *nanKETA* genes were disrupted by removing a 3.339-kb segment in the chromosomal DNA using a one-step procedure that employs PCR primers to provide the homology to the targeted sequence (Datsenko and Wanner, 2000). The deletion generates a scar in the *nan* operon that may theoretically result in the expression of the first 137 amino acids of *nanA* – the scar sequence generates a stop codon after this. The resulting peptide has been analyzed using the AllergenOnline database (www.allergenonline.com) for potential allergenicity with no sequence similarity to known allergens, independent of the method used (full sequence, sliding windows of 80 mers and 8 mers). After the deletion of *nanKETA* the strain was called ZLK.

4. lacA deletion

The *lacA* gene encodes a galactoside *O*-acetyltransferase which was shown to acetylate galactose residues in oligosaccharides produced in *E. coli* (Dumon *et al.*, 2006). Knockout of *lacA* was performed to prevent this acetylation reaction. LacA is believed to act as a detoxifying enzyme against non-metabolizable β-galactosides. As a result of the lacA knockout, the strain may theoretically be more sensitive to toxic non-metabolizable β-galactoside derivatives.

To construct the stain ZLKA from strain ZLK, the *lacA* gene was disrupted by removing a 0.513 kb segment in the chromosomal DNA using the procedure of Datsenko and Wanner

(2000). The disruption of *lacA* leaves behind the same 84 bp scar sequence as in the case of the *nanKETA* disruption. The scar generated by the deletion may theoretically result in the expression of a 62 amino acid peptide. The resulting peptide has been analyzed using the AllergenOnline database (www.allergenonline.com) for potential allergenicity with no sequence similarity to known allergens, independent of the method used (full sequence, sliding windows of 80 mers and 8 mers). After the deletion of *lacA* the strain was called ZLKA.

5. *melA* knockout

The *melA* gene encodes an α-galactosidase. As mentioned previously strain MDO is a general "platform host strain" used as a starting point to generate derived strains for the production of a large diversity of human milk oligosaccharides and other bioactive oligosaccharides. The knockout of *melA* was carried out to prevent the hydrolysis of oligosaccharides containing a terminal α-linked galactose. As a result of this knockout, the strain cannot grow on melibiose.

6. *wcaJ* knockout

The *wcaJ* gene encodes the colanic biosynthesis UDP-glucose lipid carrier transferase. Colanic acid is an extracellular polysaccharide which contains fucose and its overproduction increases dramatically the viscosity of the culture medium. The *wcaJ* knockout was performed to prevent the biosynthesis of colanic acid to reduce the culture medium viscosity and to prevent the consumption of the fucosyl donor which could compete with the formation of 2'-*O*-fucosyllactose. As a result of this deletion, the strain is assumed to have a reduced ability to form biofilms and to tolerate desiccation. This strain was called ZW.

7. *mdoH* knockout

The *mdoH* gene encodes a glucosyltransferase involved in the biosynthesis of periplasmic glucans (membrane derived oligosaccharides, MDO). At the end of the fermentation process, some of these oligosaccharides may be recovered with the HMO crude fraction. The *mdoH* knockout was performed to prevent the production of these periplasmic glucans in order to facilitate HMO purification. As a result of this deletion, the strain is assumed not to survive in medium of very low osmolarity. This strain was called MDO^- (or simply MDO). The complete genotype of the parental strain MDO is: *endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17 lacZ-wcaF::Plac nanKETA lacA melA wcaJ mdo.*

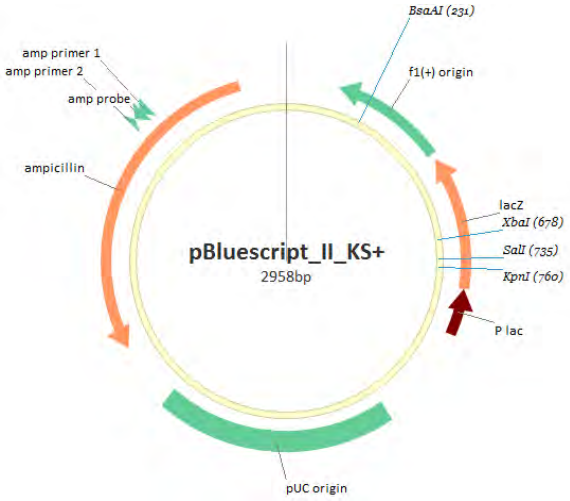
The resulting strain, MDO, constitutes a general platform starting strain for the generation of specific strains for the fermentative synthesis of a diverse range of oligosaccharides. In order to enable the specific synthesis of 2'-FL, the MDO strain was transformed with two different plasmids carrying the required genes for 2'-FL biosynthesis. The resulting strain was called SCR6, and both strains (MDO and SCR6) have been deposited at the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) in Braunschweig, Germany.

II.B.1.3 Plasmid Vectors

DNA sequences from donor organisms were inserted into either a pBS (pBluescript II KS) or pBBR1-MCS3 vector in the construction of the plasmids pBS-plasmid1 and pBBR3-plasmid2, respectively.

pBluescript II KS + is a commercial vector which was developed by Stratagene. It has the pUC origin of replication which is derived from the pBR322 pMB1 replicon. Due to the deletion of the *rop* gene it is maintained at a very high copy (several hundred copies per cells). It carries the resistance for ampicillin[1] and contains a Plac promoter.

Map of pBluescript II KS +

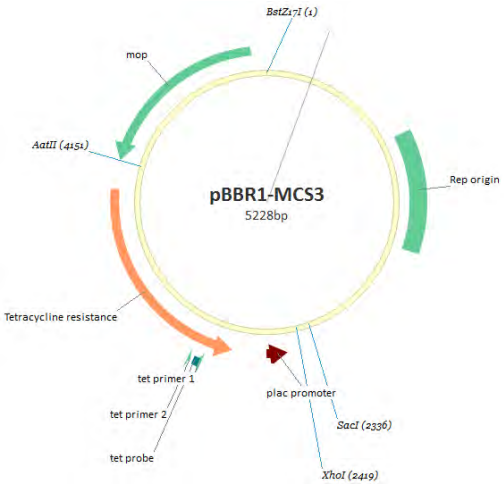


Component	Position on plasmid	Function
F1 (+) origin	135-441	f1 filamentous phage origin of replication that allows for the recovery of the SENSE strand of the *lacZ* gene when a host strain containing the pBluescript II phagemid becomes co-infected with the helper phage.
lacZ (β-galactosidase α-fragment)	460-816	This region of the lacZ gene provides α-complementation for the blue/white selection of recombinant plasmids.
Lac promoter	817-938	An inducible lac promoter upstream from the *lacZ* gene permits fusion protein expression with the β-galactosidase gene product.
pUC origin	1200-1813	Plasmid origin of replication used in the absence of helper phage. This origin will result in a copy number of 500-700 plasmids/cell.
Ampicillin resistance ORF	1976-2833	Ampicillin resistance gene for antibiotic selection of the vector
Amp primer 1	2595-2615	DNA primer used for validation of absence of DNA in product.
Amp primer 2	2554-2571	DNA primer used for validation of absence of DNA in product.
Amp probe	2573-2593	DNA probe for validations of absence of DNA in product.

[1] It is noted, however, that no antibiotic selection is used during the production of 2'-FL.

The plasmid pBBR1-MCS3 was constructed by Kovach *et al.* (1995). It is a derivative of the pBBR1 plasmid originally isolated from *Bordetella bronchiseptica* which is maintained at roughly 20 to 30 copies per cell (Antoine and Locht, 1992). The vector encodes a replication protein (Rep) that shares sequence homology with replication proteins in gram-negative bacteria. It also carries a mobilization (mob) region encoding a putative protein that shares sequence homology to Mob/Pre proteins of gram-positive plasmids. Compatibility studies involving pBBR1 suggest that this plasmid is not a member of the broad-host-range IncC, IncP, IncQ, or IncW plasmids, and it may therefore represent a novel incompatibility group.

Map of pBBR1-MCS3



Component	Position on plasmid	Function
Rep origin	909-1571	Plasmid origin of replication used in the absence of helper phage. This origin will result in a copy number of 10-40 plasmids/cell.
Lactose promoter	2492-2610	An inducible lac promoter.
Tetracycline resistance ORF	2798-3988	Tetracycline resistance gene for antibiotic selection of the vector .
Tet primer 1	2933-2954	DNA primer used for validation of absence of DNA in product.
Tet probe	2956-2979	DNA probe used for validation of absence of DNA in product.
Tet primer 2	2981-3000	DNA primer used for validation of absence of DNA in product.
mob	4151-5143	Required for mobilization

II.B.1.4 Introduced Donor Genes for 2'-FL Biosynthesis

DNA sequences from donor organisms were inserted into either the pBS or pBBR1-MCS3 vectors to create the plasmids pBS-futC-scrBR and pBBR3-GMAB-scrYA, respectively. Details on the function of the inserted DNA sequences are summarized in Table II.B.1.4-1. Since the vectors and the DNA inserts are well characterized, no unspecified DNA is expected to be associated with the genes to be transferred. All genes involved in 2'-FL biosynthesis were derived from sequences originating from *Helicobacter pylori* or were native to *E. coli* K-12 DH1.

Both donor strains were obtained from a recognized culture collection and have established species identities.

Table III.B.1.4-1 Summary of the Introduced Enzyme Activities	
Designation	**Function**
Gene1	α-1,2-fucosyl-transferase
Gene2	Phospho-mannomutase
Gene3	Mannose-1-P-guanosyltransferase
Gene4	GDP-mannose-4,6- dehydratase
Gene5	GDP-fucose synthase

II.B.1.5 Synthetic Donor Genes for Sucrose Metabolism

The plasmids also carry the genes of the sucrose operon, which enable the strain to grow on sucrose as the sole carbon source (Table II.B.1.5-1). By splitting these genes onto both plasmids, and growing the cells on sucrose medium, it is ensured that plasmid loss during fermentation is minimized. The resulting strain SCR6 is highly stable, reliable, and yields extraordinarily high titers of 2'-FL. The high production efficiency of the strain SCR6 is beneficial for product purification (*i.e.*, increasing the ratio of product to potential impurities) at the start of the downstream purification process.

As intermediates to the final strain, a synthetic DNA sequence containing the genes encoding for sucrose porin and for a PTS system sucrose-specific transporter subunit were synthesized and introduced in a pUC57 vector. A second DNA sequence containing 2 genes encoding for sucrose-6-P hydrolase and a sucrose operon repressor were also synthesized and introduced in a pUC57 vector. The two pUC57 intermediate plasmids were then used to introduce and distribute the sucrose operon over the 2 plasmids of the final strain.

Table III.B.1.5-1 Summary of the Introduced Enzyme Activities and Transporters Related to the Sucrose Operon	
Designation	**Function**
Gene6	Encodes for PTS system sucrose-specific component
Gene7	Encodes for sucrose-6-phosphate hydrolase
Gene8	Encodes for sucrose repressor
Gene9	Encodes for sucrose porin

All introduced genes comprising the sucrose operon were generated synthetically from open reading frame sequences of the annotated genomes of the donor strains, *Klebsiella pneumoniae* and *Salmonella* Typhimurium. Select modifications (silent mutations) in these sequences were introduced to remove inherent restriction sites. As the introduced genes are synthetic in origin and have well characterized functions (*i.e.*, sucrose metabolism) the potential

introduction of undesirable genes from the donor organisms, which are members of pathogenic/toxicogenic species, is avoided.

II.B.2 Raw Materials and Processing Aids

The raw materials used as carbon sources for 2'-FL biosynthesis include D-lactose and D-sucrose. These sugars, which meet or exceed food grade quality standards (*i.e.*, European Pharmacopoeia) are sterilized before use and are subject to quality control pre-screening using High-Performance Liquid Chromatography (HPLC) for purity (*e.g.*, lactose can isomerize to lactulose through the Lobry de Bruyn–van Ekenstein aldose-ketose isomerization). Fermentation is performed in a chemically-defined, salt-based, minimal growth medium that excludes the use of antibiotics. Induction of 2'-FL biosynthesis requires the addition of small amounts of IPTG.

Fermentation and other processing-aids and filters and filter aids used in the production of 2'-FL are food grade quality[2] and are used in accordance with an applicable federal regulation, previous GRAS determination, and/or have been the subject of an effective food contact notification (see Table II.B.2-1).

Table II.B.2-1 Raw Material and Processing Aids Used in the Manufacture of 2'-FL by Fermentation

Material	Function
Raw Material Substrates (Bioreagents)	
D-sucrose	Carbon and energy source
D-lactose	Substrate/source raw material
Other Components of Fermentation Medium	
Ammonium hydroxide	Fermentation medium ingredient
Magnesium sulfate	Fermentation medium ingredient
Ammonium dihydrogen phosphate	Fermentation medium ingredient
Potassium dihydrogen phosphate	Fermentation medium ingredient
Potassium hydroxide	Fermentation medium ingredient
Sodium hydroxide	Fermentation medium ingredient
Citric acid	Fermentation medium ingredient
Thiamine	Fermentation medium ingredient
Processing Aids	
Acetic acid	Crystallization
Isopropyl-β-D-1-thiogalactoside	Induces expression of enzyme
Polypropylene glycol	Anti-foaming

[2]Compliant with the specifications set forth in the Food Chemicals Codex or equivalent international food or pharmacopeia standard.

Table II.B.2-1 Raw Material and Processing Aids Used in the Manufacture of 2'-FL by Fermentation	
Material	**Function**
Filter and Filter Aids	
Ultrafiltration	Removal of cell matter and proteins
Nanofiltration	Removal of small molecules
Ion-exchange resin	Removal of charged molecules
Electrodialysis	Removal of small charged molecules (*e.g.*, salts)
Charcoal filter	Decolorization and removal of impurities
Microfiltration (0.2 µm)	Filter sterilization

II.B.3 Manufacturing Process

The manufacturing process for 2'-FL can be broadly divided into 2 stages, which are described in brief in Section II.B.2.1 and II.B.2.2, respectively (see Figure II.B.3-1 below).

Figure II.B.3-1 Schematic of the Overall Manufacturing Process for 2'-FL

Stage	Step No	PROCESS STEP	PURIFICATION
Upstream Processing (USP)	01	Media Preparation	
	02	Propagation	
	03	Seed Fermentation	
	04	Fermentation	*Production of 2'-FL*
	05	Ultrafiltration (UF/DF)	*Removal of cells and large biomolecules (e.g. protein, nucleic acids and lipopolysaccharides)*
Downstream Processing (DSP)	06	Nanofiltration (NF)	*Concentration. Reduction water, minerals and very small biomolecules*
	06a	*Optional Microfiltration*	*Removal of potential microbiological contamination*
	06b	*Optional Ion Removal (e.g., .ion-exchange resin or electrodialysis)*	*Removal of small charged molecules and salts (e.g. trace metals)*
	06c	*Optional Pre-concentration (e.g., evaporation or nanofiltration)*	
	07	Decoloration (*e.g.*, charcoal filtration)	*Removal of color and impurities by adsorbent*
	08	Microfiltration	*Removal of potential microbiological contamination*
	09	Pre-concentration (*e.g.,* evaporation or nanofiltration)	
	10	Crystallization (from water with acetic acid)	*Highly efficient removal of micro-impurities (traces of protein and DNA, amino acids, carbohydrate-type impurities, trace elements, etc.)*
	11	Solid-Liquid-Separation (SLS)	
	12	Washing	
	13	Drying	*Removal of water and acetic acid*

Figure II.B.3-1	Schematic of the Overall Manufacturing Process for 2'-FL	
14	Sampling and Packaging	
15	Quality Control	*Parameters of specifications are tested and CoA issued*
16	Batch Release	

II.B.3.1 Manufacturing Stage 1: Fermentation Procedure

Fermentation is performed in a chemically-defined, salt-based, minimal medium, with D-sucrose as the only carbon source and D-lactose as the substrate. No antibiotics or chelators (*e.g.*, nitrilotriacetic acid) are used during fermentation. The major constituents of the fermentation medium are D-sucrose, D-lactose, ammonium hydroxide, magnesium sulfate, ammonium dihydrogen phosphate, potassium dihydrogen phosphate, potassium hydroxide, sodium hydroxide and citric acid. The expression of the enzymes required for 2'-FL production is induced by the addition of small amounts of IPTG during the induction phase and the fermentation is maintained for several days until in-process quality controls indicate a favorable ratio of 2'-FL to other carbohydrates as well as high consumption of D-lactose.

2'-FL is efficiently excreted into the fermentation broth, therefore disruption of the cells is not required for isolation of 2'-FL from the culture broth. The microbial biomass containing the intact production organism is then removed from the culture supernatant containing 2'-FL by a 15 kD ultrafiltration/diafiltration aid and the separated microbial biomass is deactivated by heat treatment. The quality of the clear ultrafiltration/diafiltration permeate is assessed by a range of in-process quality controls and then further purified in the second stage of the production process, the downstream processing (see Section II.B.3.2 below).

II.B.3.2 Manufacturing Stage 2: Purification and Isolation

Stage 2 of the manufacturing process consists of a series of purification steps, most notably the final selective crystallization step, that generates the single, isolated, high-purity, crystalline trisaccharide 2'-FL ingredient.

The ultrafiltration permeate is concentrated through a nanofiltration aid to remove water, minerals, and very small molecules. Depending on processing time of the nanofiltration, an optional microfiltration step can be applied in order to minimize the risk of microbiological growth, and an optional ion removal step can be applied by treatment with an ion exchange resin or by performing electrodialysis. If ion removal is performed, the solution will need to be pre-concentrated before proceeding to the next step in the purification process. Subsequently, decoloration of the solution is achieved by treatment with an adsorbent (*e.g.*, activated charcoal), and the solution is microfiltered to minimize the risk of microbial growth. The pH of the solution is controlled and adjusted if necessary with sodium hydroxide to reduce the risk of 2'-fucosyllactulose formation *via* the pH-dependent Lobry de Bruyn–van Ekenstein aldose-keto

isomerism during concentration. The solution is then pre-concentrated to a defined concentration of 2'-FL either by vacuum distillation or nanofiltration. The highly-controlled addition of defined amounts of acetic acid and 2'-FL seeding crystals in defined intervals of time and temperature leads to the crystallization of 2'-FL. A solid-liquid-separation removes the 2'-FL crystals from the mother liquor[3], and the crystals then are washed with acetic acid to remove remaining traces of salts, biomolecules, and carbohydrate impurities, then dried until specifications for acetic acid are met. Quality control measures are in place during the entire purification and isolation process to ensure that final batches of 2'-FL released conform to the product specifications.

The 2'-FL produced by fermentation is identical to 2'-FL present in human milk from lactating women and also to the chemically-synthesized 2'-FL described previously (U.S. FDA, 2014a). There have been no modifications to the molecular structure of 2'-FL during its manufacture from that of the 2'-FL present in human milk.

II.B.4 Quality Control

Both manufacturing stages are controlled by a HACCP plan which includes specifications for the equipment, raw materials, product, and packaging materials used in the manufacturing process. Master operating instructions are followed, batch records are kept, a number of in-process controls are applied, and the final isolated 2'-FL product is controlled by Certificates of Analyses and batch release routines.

II.C Specifications for Food Grade Material and Product Analysis

II.C.1 Specifications for Food Grade Material

Food-grade specifications have been established for 2'-FL and are presented in Table II.C.1-1 below. Specifications established for potential impurities and contaminants and microbial endotoxins are further discussed in the sections that follow. All methods of analysis are nationally or internationally recognized or have been validated by Glycom or a third party laboratory.

[3] The solution that is removed by filtration from the crystallized product is called the "mother liquor". It contains potential impurities and traces of product.

Table II.C.1-1 Proposed Product Specifications for 2'-FL		
Parameter	**Specification**	**Method**
Appearance	Powder or agglomerates	MSZ ISO 6658:2007
Color	White to off white	MSZ ISO 6658:2007
Identification	RT of main component corresponds to RT of standard ± 3%	Glycom method HPLC-202-2C4-002
2'-FL Assay by HPLC (water free)	Min. 94.0%	Glycom method HPLC-202-2C4-002
D-Lactose	Max. 3.0 w/w%	Glycom method HPLC-206-2C4-001
L-Fucose	Max. 1.0 w/w%	Glycom method HPAEC-206-001
Difucosyllactose	Max. 1.0 w/w%	Glycom method HPAEC-206-001
Total Human-identical milk oligosaccharides[a]	Min 96.0%	Glycom methods HPLC-202-2C4-002, HPLC-206-2C4-001 and HPAEC-206-001
2'-Fucosyl-D-lactulose	Max. 1.0 w/w%	Glycom method HPLC-206-2C4-001
pH (20°C, 5% solution)	3.2 to 5.0	Ph. Eur. 2.2.3
Water	Max. 5.0%	Karl-Fischer (Ph. Eur. 2.5.32)
Ash, sulfated	Max. 1.5%	Ph. Eur. 6.7 04/2010:20414
Acetic acid (as free acid and/or sodium acetate)	Max. 1.0%	Megazyme K-ACETRM 07/12
Residual proteins	0.01%	Bradford Assay; Glycom method UV-001
Heavy metals		
Lead	Max 0.1 mg/kg	ICP-MS by EPA 6020A:2007
Microbiological Parameters		
Salmonella	Absent in 25 g	MSZ-EN-ISO 6579:2006
Aerobic mesophilic total plate count	Max. 500 CFU/g	MSZ-EN-ISO 4833-1:2014
Enterobacteriaceae	Absent in 10 g	ISO 21528-1:2004, MSZ ISO 21528-2:2007
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	MSZ-EN-ISO 11290-1:1996/A1:2005, MSZ EN ISO 11290-1:1998
Bacillus cereus	Max. 50 CFU/g	MSZ-EN-ISO 7932:2005
Yeasts	Max. 10 CFU/g	MSZ-ISO 7954:1999
Molds	Max. 10 CFU/g	MSZ-ISO 7954:1999

2'-FL = 2'-*O*-fucosyllactose; CFU = colony forming units; Eur. Ph. = European Pharmacopeia; EU = endotoxin units; HPAEC = high-performance anion-exchange chromatography; HPLC = high performance liquid chromatography; ISO = International Organization for Standardization; RT = retention time.
[a] Human-identical milk oligosaccharides is defined as the sum of 2'-FL, lactose, difucosyllactose, and fucose.

II.C.2 Product Analysis

Batch analyses for 4 independent commercial batches supporting the product specifications laid out in Section II.C.1 are presented in Table II.C.2-1.

Table II.C.2-1 Batch Analyses for 2'-FL Produced by Fermentation

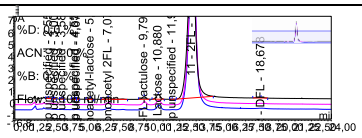
Parameter	Specification	Manufacturing Batch Number			
		Batch (b) (4)	Batch (b) (4)	Batch (b) (4)	Batch (b) (4)
Appearance	Powder or agglomerates	Complies	Complies	Complies	Complies
Color	White to off white	Complies	Complies	Complies	Complies
Identification	RT of main component corresponds to RT of standard ± 3%	Complies	Complies	Complies	Complies
2'-FL Assay by HPLC (water and solvent free)	Min. 94.0%	97.6%	98.4%	97.2%	98.4%
D-Lactose	Max. 3.0 w/w%	0.6%	0.57%	0.57%	0.84%
L-Fucose	Max. 1.0 w/w%	< 0.03%	0.03%	0.03%	<0.03%
Difucosyllactose	Max. 1.0 w/w%	0.21%	0.12%	0.05%	0.08%
Human-identical milk oligosaccharides[a]	Min 96.0%	98.4%	99.1%	97.9%	99.3%
2'-Fucosyl- D -lactulose	Max. 1.0 w/w%	0.28%	0.23%	0.34%	0.32%
pH (20°C, 5% solution)	3.2 to 5.0	4.2	4.1	3.9	3.8
Water	Max. 5.0%	0.47%	0.35%	0.38%	0.23%
Ash, sulfated	Max. 1.5%	0.62%	0.69%	0.77%	0.77%
Acetic acid (as free acid and/or sodium acetate)	Max. 1.0%	0.20%	0.25%	0.32%	0.41%
Residual proteins	0.01%	<LOD[b]	<LOD[c]	<LOD[b]	<LOD[b]
Heavy metals					
Lead	Max 0.1 mg/kg	<0.1	<0.1	<0.1	<0.1
Microbiological Parameters					
Salmonella	Absent in 25 g	Complies	Complies	Complies	Complies
Aerobic mesophilic total plate count	Max. 500 CFU/g	Complies	Complies	Complies	Complies
Enterobacteriaceae	Absent in 10 g	Complies	Complies	Complies	Complies
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Complies	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25 g	Complies	Complies	Complies	Complies
Bacillus cereus	Max. 50 CFU/g	Complies	Complies	Complies	Complies
Yeasts	Max. 10 CFU/g	Complies	Complies	Complies	Complies
Molds	Max. 10 CFU/g	Complies	Complies	Complies	Complies

2'-FL = 2'-*O*-Fucosyllactose; CFU = colony forming units; EU = endotoxin units; LOD = limit of detection; w/w = weight/weight.
[a] Human-identical milk oligosaccharides is defined as the sum of 2'-FL, lactose, difucosyllactose, and fucose.
[b] The LOD is 0.0017% (w/w).
[c] The LOD is 0.0005 % (w/w).

II.C.3 Additional Quantitative and Qualitative Analyses

As discussed, 2'-FL produced by fermentation with *Escherichia coli* (*E. coli*) K-12 SCR6 is chemically and structurally identical to the 2'-FL produced by chemical synthesis methods described in GRN 546 and to 2'-FL that is present in human breast milk, as confirmed by ^{1}H- and 2D-NMR-spectroscopy, mass spectrometry and x-ray crystallography. Batch analyses from multiple lots of 2'-FL produced by fermentation have been analyzed using High-Performance Liquid Chromatography (HPLC), High-Performance Anion Exchange Chromatography (HPAEC) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). All batches displayed as similar impurity profile demonstrating consistency of the manufacturing process. Figure II.C.3-1 shows a representative impurity profile of several batches as measured by HPLC using a Corona Charged Aerosol Detector (cCAD). The main advantages of the detector used are high sensitivity, wide dynamic detection range, consistent performance with excellent precision and more consistent response over wide range of chemical structures (*e.g.*, no chromophores are required for detection).

Figure II.C.3-1 Representative Impurity Profile of 2'-FL as Measured by High Performance Liquid Chromatography and Corona Charged Aerosol Detector



Results of the HPLC-cCAD analyses demonstrate that saccharides that are structurally related to 2'-FL can be present at small quantities in the final isolated product, namely D-lactose (not more than 3%), L-fucose (not more than 1%), difucoslylactose (not more than 1%) and 2'-fucosyl-D-lactulose (not more than 1.0%). The first three (lactose, fucose and difucosyllactose) are all natural components of breastmilk and the resulting exposure from the levels in 2'-FL produced by fermentation would be insignificant compared to the exposure from each of these saccharides at the naturally occurring levels.

2'-Fucosyl-D-lactulose is an isomerization product of 2'-FL where the terminal glucose moiety is converted into a fructose sugar. This type of isomerization is pH and temperature dependent

and has been commonly reported for the closely related conversion of D-lactose into D-lactulose during heat treatment [*i.e.*, ultra-high temperature (UHT) processing and pasteurization] of milk, including human donor milk (Beach and Menzies, 1983; Schuster-Wolff-Bühring *et al.*, 2010; Gómez de Segura *et al.*, 2012). This isomerization reaction of carbohydrates is also known as the Lobry de Bruyn–van Ekenstein transformation (Angyal, 2001; Wang, 2010). Different infant formulas have been reported to contain D-lactulose at relative levels between 1 and 7% of their D-lactose content, and absolute levels up to 13.7 mmol/L (Beach and Menzies, 1983). Although the isomerization product of 2'-FL has not been specifically reported in heat treated human donor milk, D-lactulose has also here been detected at significant proportions of D-lactose (Gómez de Segura *et al*., 2012), and it can thus be reasonably assumed that 2'-fucosyl-D-lactulose is present at comparable ratios and can thereby be equally regarded to have a history of safe use from heat treated human donor milk. In any case, at the low levels permitted in 2'-FL (not more than 1.0 %) any effects of this structurally very closely related by-product can be neglected.

II.C.4 Manufacturing Impurities and Contaminants

II.C.4.1 Amino Acids and Biogenic Amines

Although 2'-FL is harvested from the fermentation medium without disruption of the cells, batch analyses also included a variety of HPLC based methods for secondary metabolites and other cell derived impurities that may originate from the fermentation medium. Various theoretical cell metabolism impurities produced during fermentation (*e.g.*, glutamic acid, GABA, histamine, tyramine, spermidine, cadaverine and putrescine) were not present at detectable levels in the final ingredient using sensitive HPLC based analyses methods (data not shown).

II.C.4.2 Microbial Endotoxins and Residual Proteins

Internal specifications for lipopolysaccharides (*i.e*., endotoxins) originating from the fermentation organism have been established as an additional quality control point to ensure that microbial endotoxins are efficiently removed and/or not introduced during the production process. Regulatory threshold levels for food regarding endotoxin contamination currently do not exist. Typical ranges of endotoxin load have been reported for drinking water (O'Toole *et al.*, 2008; Anderson *et al.*, 2002), cow's milk (Gehring *et al.*, 2008), and infant formula powder (Townsend *et al.*, 2007). The endotoxin specification for 2'-FL is set to not contribute additional exposure to endotoxins that would result in exposures above the usual levels that are expected for infant formula powder currently on the market (Townsend *et al.*, 2007). The endotoxin specification established for 2'-FL is assayed using the *Limulus* amebocyte lysate kinetic chromogenic assay described in the European Pharmaceopoeia. Batch analyses of 2'-FL demonstrate compliance to the endotoxins specifications.

Glycom also notes that the transfer of residual protein from the fermentation process into the finished 2'-FL ingredient is significantly reduced by the use of ultrafiltration, adsorption (*e.g.*, activated carbon treatment) and multiple crystallization/wash steps employed during the manufacturing process. 2'-FL is efficiently secreted from the cell during fermentation and therefore can be isolated from the culture broth without disruption of the cells, which further reduces the potential to introduce proteins or other intracellular metabolites to the ingredient. All batches of 2'-FL are specified to contain <0.01% protein as analyzed using a modified Bradford method.

II.C.4.3 Absence of Production Organism and Introduced Antibiotic Resistance Genes

The production microorganism is efficiently removed by the ultrafiltration step during upstream processing which is applied directly after fermentation. Additionally, during downstream processing, various sequential purification processes are also applied to ensure microbiological purity.

The absence of the microorganisms in the ingredient is demonstrated by microbial testing for *Enterobacteriaceae* and *Escherichia coli* during batch analyses according to internationally-recognized methods (ISO 21528-1:2004, MSZ ISO 21528-2:2007, ISO 7251:2005). The absence of the production organism in the ingredient is also supported by the analysis of residual DNA in batches of the final ingredient. The absence of residual DNA from the production organism is confirmed by 3 different validated quantitative PCR (qPCR) methods. These qPCR methods target short subsequences of the antibiotic resistance marker genes *ampR* and *tetR* (located on the high-copy plasmids) and a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. Analysis of 4 batches of 2'-FL demonstrate no detectable levels of residual DNA (limit of quantification = 5 ppb) present in the final ingredient.

II.C.4.4 Minerals and Lactose

Small amounts of minerals and lactose originating from the fermentation media are expected to be present in 2'-FL; however, as shown in Table II.C.4.4-1, these concentrations result in quantitatively insignificant carry-over into the finished infant formula.

Table II.C.4.4-1 Mineral and Lactose Contribution of 2'-FL to Infant Formula			
Nutrient	**Mean Results from 4 Lots of 2'-FL Produced by Fermentation**	**Nutrient Value Provided by a Use-Level of 2.4 g/L 2'-FL[a]**	**Nutrient Requirements for Infant Formula under §350a of the FFDCA**
Minerals	*(mg/kg)*	*(mg/100 kcal)*	*(mg/100 kcal)*
Phosphate[b]	3	0.001	25
Chloride	≤40	0.0016	55 - 150
Sodium (Na)	232	0.093	20 - 60
Potassium (K)	163	0.065	80 - 200
Magnesium (Mg)	826	0.331	6
Calcium (Ca)	648	0.260	50
Iron (Fe)	3.5	0.001	0.15
Zinc (Zn)	< 2	< 0.001	0.5
Copper (Cu)	< 1	< 0.0004	60
Manganese (Mn)	1.25	0.0005	5
Lactose	0.65 (% dry weight basis)	0.0026 g (2.6 mg)	-

[a] Values calculated based upon the assumption that 167 mL of infant formula provides approximately 100 kcal.
[b] Phosphate as orthophosphate

II.D Stability

II.D.1 Bulk Stability

The bulk stability of 2'-FL produced by fermentation has been confirmed under accelerated conditions at 60°C and ambient humidity over a 6-month storage period and under accelerated conditions at 80°C and ambient humidity over a 3-month storage period. The data from this study is presented in Table II.D.1-1. 2'-FL was stable throughout the 6-month storage period with no measureable loss of 2-'FL or change in impurity content. Based on these studies the stability of the ingredient produced by fermentation is calculated to be at least 5 years when protected from light and stored at room temperature and ambient humidity. A 2-year accelerated stability study and a real-time 5-year stability study is currently ongoing on representative batches of 2'-FL.

Table II.D.1-1 Accelerated Stability Study on 2'-*O*-Fucosyllactose (2'-FL) (60°C and 80°C; ambient humidity) (Batch (b) (4))					
Parameter Tested	**Analytical Data**				
	Time 0	**2 Weeks**	**1 Month**	**3 Months**	**6 Months**
60°C, Ambient Humidity					
Sensory	Almost white powder with moderate acetic odor	Almost white powder with slight acetic odor	Almost white powder with slight acetic odor	Almost white powder with slight acetic odor	Almost white powder with slight acetic and caramel odor
Assay (% w/w)	94.7	NA	NA	96.1	NA
Water (% w/w)	0.30	0.55	NA	0.50	0.35
Acetic acid (% w/w)	0.30	NA	NA	0.29	0.24

Table II.D.1-1 Accelerated Stability Study on 2'-*O*-Fucosyllactose (2'-FL) (60°C and 80°C; ambient humidity) (Batch (b) (4))					
Parameter Tested	**Analytical Data**				
	Time 0	**2 Weeks**	**1 Month**	**3 Months**	**6 Months**
Impurities (% w/w)					
Fucose	< 0.03	< 0.03	< 0.03	< 0.03	< 0.03
Lactose	2.15	2.11	2.08	2.07	2.07
2'-Fucosyllactulose	0.18	0.18	0.19	0.19	0.20
DFL	0.18	0.17	0.17	0.16	0.16
Total unidentified impurities	0.06	0.05	0.05	0.05	0.05
80°C, Ambient Humidity					
Sensory	NA	NA	Almost white powder with neutral odor	Almost white powder with slightly caramel odor	NA
Assay (%w/w)	NA	NA	NA	94.5	NA
Water (%w/w)	NA	NA	NA	0.54	NA
Acetic acid (%w/w)	NA	NA	0.25	0.23	NA
Impurities (%w/w)					
Fucose	NA	NA	< 0.03	< 0.03	NA
Lactose	NA	NA	2.04	2.01	NA
2'-Fucosyllactulose	NA	NA	0.20	0.20	NA
DFL	NA	NA	0.14	0.12	NA
Total unidentified impurities	NA	NA	0.04	0.04	NA

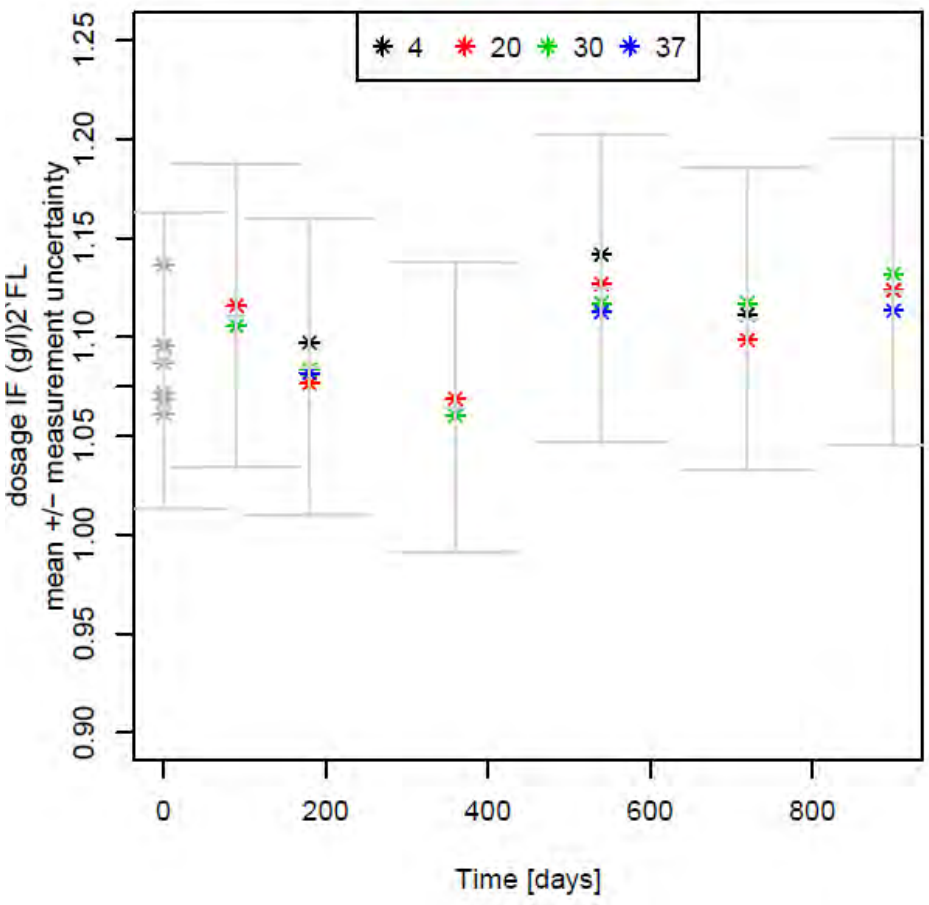
2'-FL = 2'-*O*-fucosyllactose; DFL = difucosyllactose; NA = not analyzed.

II.D.2 Stability Under the Intended Conditions of Use

II.D.2.1 Stability in Powdered Infant Formula

The stability of 2'-FL in combination with lacto-*N*-neotetraose (LNnT) in infant formula has been previously investigated in long-term studies. These studies are discussed in detail by Glycom in Section II.D.2 of GRN 546 (U.S. FDA, 2014ac). Briefly, three independently formulated, commercially representative infant formula powders containing a target concentration of 0.90 g 2'-FL and 0.45 g LNnT per 100 g (dry matter) of infant formula respectively were subjected to typical production processing steps and stored in gassed (N_2/CO_2) tin cans (1 can per time and temperature point) at a temperature of 4, 20, 30, or 37°C. 2'-FL content was measured at regular time intervals for up to 900 days of storage. No significant loss of 2'-FL was observed under any of the storage conditions. The results from one production batch are presented in Figure II.D.2.1-1 below.

Figure II.D.2.1-1 Concentration of 2'-FL in a Commercially Representative Infant Formula Following Storage for up to 900 Days at Increasing Temperatures



As 2'-FL produced by fermentation is compositionally comparable to chemically-synthesized 2'-FL, there are no anticipated differences in their stabilities in food matrices. As such, the study summarized above supports the stability of 2'-FL produced by fermentation under its intended conditions of use in infant formula.

II.D.2.2 Stability in Other Food Applications

The stability of chemically-synthesized 2'-FL also has been evaluated in other conventional food matrices, including yogurts, ready-to-drink flavored milk, and citrus fruit beverages, the results of which are detailed in Section II.D.2 of the previous GRAS notification (U.S. FDA, 2014a). All stability studies were conducted using formulations representative of commercial food products on the market and under typical processing[4] and storage conditions for such products. The analytical results from these studies demonstrate that there were no losses of 2'-FL during the processing steps or during storage.

[4] Typical processing steps include pasteurization and/or ultra-high temperature heating.

As 2'-FL produced by fermentation is considered compositionally comparable to chemically-synthesized 2'-FL, there are no anticipated differences in their stabilities in food matrices. As such, the study summarized above supports the stability of 2'-FL produced by fermentation under its intended conditions of use in conventional foods.

III. SELF-LIMITING LEVELS OF USE

No known self-limiting levels of use are associated with Glycom's 2'-FL ingredient.

IV. BASIS FOR GRAS DETERMINATION

The determination that 2'-FL, as described herein, is GRAS under the intended conditions of use in non-exempt term infant formula and in conventional food and beverage products as described in Section I.D.1 is on the basis of scientific procedures. The totality of generally available data relevant to the safety of 2'-FL for use in term infant formula and various conventional food and beverage products have been the subject of comprehensive systematic reviews by qualified experts (U.S. FDA, 2014a,b), and by authoritative bodies including the FDA (U.S. FDA, 2015c,d) and the European Food Safety Authority (EFSA) (EFSA, 2015a). Published studies relevant to the safety of 2'-FL for use in infant formula and conventional food products, which include studies characterizing the background consumption of 2'-FL from human milk, information on the metabolic fate of 2'-FL, toxicological studies, and safety and tolerance in humans, have been incorporated by reference to previous GRAS determinations (U.S. FDA, 2014a,b). A summary of the history of safe consumption, case of need in infant formula, metabolic fate, toxicology and human safety of 2'-FL is provided in Sections IV.A through IV.E. An updated literature search was conducted to identify any new data and information relevant to the safety of 2'-FL published since the previous GRAS determinations. New studies identified in the literature did not report physiological or potentially toxicological effects of 2'-FL to suggest that the intended uses of 2'-FL as a food ingredient may be unsafe. A discussion on the allergenic risk of 2'-FL produced by fermentation is provided in Section IV.F; information on the safety of the production strain and associated biotechnological modifications also are discussed.

The data and information summarized below were reviewed by a Panel of Experts, qualified by scientific training and experience to evaluate the safety of 2'-FL produced by microbial fermentation, who concluded that 2'-FL is GRAS under the aforementioned conditions of intended use in infant formula and in food based is on the basis of scientific procedures.

IV.A Probable Consumption

Dietary Intake of 2'-FL among Infants and Toddlers

2'-FL manufactured by Glycom using fermentation is intended for use in non-exempt term infant formula, and baby foods, including toddler formula (Table I.D-1). Potential dietary exposures to 2'-FL among infant and toddler consumers of term infant formula and baby foods to which 2'-FL may be added have been evaluated previously, and the reader is therefore directed to sections IV.A.1 and IV.A.2 of GRN 546 for detailed summaries of the stratified assessment of 2'-FL intake by this population group. Considering that 2'-FL produced by fermentation will serve as an alternative to other 2'-FL sources that have previously been determined to be GRAS (*i.e.*, GRN 546, 571) and is intended for addition to term infant formula and baby foods at the same use levels, the proposed uses of 2'-FL, manufactured by fermentation, will not increase dietary exposures in this population group.

Dietary Intake in General U.S. Population from all Proposed Food Uses

Stratified assessments of dietary intake of 2'-FL among U.S. consumers of various conventional food and beverage products to which 2'-FL may be added have been conducted previously (*i.e.*, GRN 546). As described in Table I.D-1, the food uses of 2'-FL have been revised and no longer include applications in baked goods and baking mixes, carbonated beverages, flavored and enhanced waters, coffee and tea, beverage whiteners, fruit flavored drinks and ades, vegetable juices and nectars, and table top sweeteners. Furthermore, the use levels of 2'-FL in non-dairy yogurt and yogurt have been lowered from 10.6 g/kg to a maximum proposed use level of 5.3 g/kg. Estimates for the daily intake of 2'-FL were therefore updated to incorporate the lowered uses of 2'-FL in certain food categories as well as employ the more recently published food consumption data from the U.S. National Center for Health Statistics' (NCHS) 2011-2012 National Health and Nutrition Examination Surveys (NHANES). The following sections summarize the estimated mean and 90th percentile daily intake of 2'-FL among the U.S. population.

Estimates for the intake of 2'-FL were calculated based on the individual food uses and maximum use levels presented in Table I.D-1 in conjunction with the food consumption data included in the most recent release of NHANES (USDA, 2014; CDC, 2015). Food codes were grouped in food-use categories according to Title 21, Section §170.3 of the Code of Federal Regulations (U.S. FDA, 2015e). Product-specific adjustment factors were developed based on data provided in the standard recipe file for the Continuing Survey of Food Intakes by Individuals (CSFII) 1994-1996, 1998 survey (USDA, 2000). Estimates for the total daily intake of 2'-FL from all intended food uses are summarized in Table IV.A-1 on a per person basis by population group. Table IV.A-2 presents these data on a per kilogram body weight basis.

Approximately 85.0% of the U.S. population was identified as potential consumers of foods containing 2'-FL (designated as "users"). As a result of the high percentage of users identified within all population groups, the intake estimates for the all-person (*i.e*., all individuals surveyed) and all-user (*i.e.* consumers only) categories were similar; therefore, only the all-user results are discussed in detail.

The mean and 90th percentile intake of 2'-FL by the total U.S. population from all intended food uses was estimated to be 0.64 and 1.31 g/person/day, respectively. The total population estimated mean and 90th percentile intakes on a body weight basis were determined to be 20.0 and 33.1 mg/kg body weight/day, respectively.

Among the individual population groups, the highest mean and 90th percentile intakes of 2'-FL on both an absolute and per body weight basis were identified in toddlers. The mean and 90th percentile of intakes of 2'-FL in this population group were determined to be 1.12 g/person/day (equivalent to 84.9 mg/kg body weight/day), and 1.97 g/person/day (equivalent to 146.0 mg/kg body weight/day), respectively.

Female adults and the elderly were observed to have the lowest mean all-user intakes of 0.49 g/person/day and female adults of childbearing age had the lowest 90th percentile intakes of 1.05 g/person/day. On a body weight basis, the lowest mean intakes of 2'-FL were identified in elderly adults at 6.8 mg/kg body weight/day, whereas female adults had the lowest 90th percentile all-user intakes at 15.3 mg/kg body weight/day.

Table IV.A-1 Summary of the Estimated Daily Intake of 2'-FL from All Proposed Food and Beverage Uses in the U.S. by Population Group (2011-2012 NHANES Data)

Population Group	Age Group (Years)	All-Person Consumption (g/day)		All-Users Consumption (g/day)			
		Mean	90th Percentile	% Users	n	Mean	90th Percentile
Toddlers	1 to 3	1.11	1.96	99.3	561	1.12	1.97
Children	4 to10	0.68	1.25	98.5	1,161	0.69	1.25
Female Teenagers	11 to 18	0.45	1.03	90.0	513	0.50	1.12
Male Teenagers	11 to 18	0.58	1.19	90.2	474	0.65	1.29
Female Adults of child bearing age	19 to 40	0.42	1.02	84.7	698	0.50	1.05
Female Adults	19 to 64	0.40	0.99	81.3	1,438	0.49	1.10
Male Adults	19 to 64	0.51	1.27	79.8	1,313	0.64	1.38
Elderly Adults	65 and up	0.43	1.04	87.9	816	0.49	1.12
Total Population	All Ages	0.54	1.23	85.0	6,574	0.64	1.31

2'-FL = 2'-*O*-fucosyllactose; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

Table IV.A-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL from All Proposed Food and Beverage Uses in the U.S. by Population Group (2011-2012 NHANES Data)							
Population Group	**Age Group (Years)**	**All-Person Consumption (mg/kg bw/day)**		**All-Users Consumption (mg/kg bw/day)**			
		Mean	**90th Percentile**	**%**	**n**	**Mean**	**90th Percentile**
Toddlers	1 to 3	84.4	146.0	99.3	558	84.9	146.0
Children	4 to10	27.0	54.5	98.5	1,161	27.4	54.5
Female Teenagers	11 to 18	8.3	19.6	89.9	506	9.2	21.1
Male Teenagers	11 to 18	9.8	22.2	90.1	471	10.9	22.4
Female Adults of child bearing age	19 to 40	6.1	14.8	84.6	688	7.2	15.8
Female Adults	19 to 64	5.8	14.3	81.4	1,419	7.1	15.3
Male Adults	19 to 64	6.0	14.6	79.7	1,304	7.6	16.0
Elderly Adults	65 and up	6.0	13.9	87.9	804	6.8	15.4
Total Population	All Ages	17.0	28.7	85.0	6,523	20.0	33.1

2'-FL = 2'-*O*-fucosyllactose; bw = body weight; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

IV.B History of Safe Consumption

2'-FL is one of the naturally occurring fucosylated milk oligosaccharides present in some mammalian milks (Urashima *et al.*, 2001; Castanys-Muñoz *et al.*, 2013), with markedly the highest concentrations of 2'-FL occurring in milk from lactating women (Kuhn *et al.*, 1955). 2'-FL therefore has an established history of safe consumption by infants consuming human milk.

As discussed previously in GRN 546, the 2'-FL content of human milk has been reported in several publications from independent research groups, whereby extensive data have been provided according to secretor and Lewis-blood group status (Thurl *et al.*, 1996, 2010; Coppa *et al.*, 2011; Galeotti *et al.*, 2012, 2014), ethnicity (Erney *et al.*, 2000; Musumeci *et al.*, 2006), lactation period (Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Asakuma *et al.*, 2008; Leo *et al.*, 2010; Gabrielli *et al.*, 2011; Bao *et al.*, 2013), term/preterm birth (Nakhla *et al.*, 1999; Gabrielli *et al.*, 2011), and other studies measuring the content of mature milk (Chaturvedi *et al.*, 1997, 2001a; Nakhla *et al.*, 1999; Erney *et al.*, 2000, 2001; Sumiyoshi *et al.*, 2003; Morrow *et al.*, 2004; Leo *et al.*, 2010; Thurl *et al.*, 2010; Asakuma *et al.*, 2011; Coppa *et al.*, 2011; Galeotti *et al.*, 2012; Smilowitz *et al.*, 2013; Hong *et al.*, 2014; Balogh *et al.*, 2015). Based on Glycom's comprehensive review of the literature, a use level of 2,400 mg/L was considered representative of mean concentrations that have been reliably measured in human milk samples from full term birth mothers across a variety of demographic groups, Lewis body genotypes, and lactational stages (U.S. FDA, 2014a). Following a similar review of the published literature, Jennewein Biotechnologie reported mean concentrations of *"~2.6 g/L"* for 2'-FL in human milk

samples and established an arbitrary use level of 2 g/L for 2'-FL for term infant formula to approximate levels occurring in human milk (U.S. FDA, 2014b).

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/kg body weight/day of 2'-FL (Davies *et al.*, 1994; Hester *et al.*, 2012). Among infants from Secretor mothers, the intake of 2'-FL from mature breast milk may be up to 1,150 mg/kg body weight/day (U.S. FDA, 2014a).

For newborn infants, the average intake of 2'-FL from colostrum is approximately 80 to 360 mg/kg body weight/day based on a 3.4-kg newborn infant (WHO, 2015) drinking an average of 250 mL of breast milk per day during the first 5 days (Hester *et al.*, 2012). However, in newborns from secretor mothers, the intake of 2'-FL from colostrum may be up to approximately 620 mg/kg body weight/day (U.S. FDA, 2014a).

IV.C Case-of-Need for Infant Formula

2'-FL is the most abundant oligosaccharide member of the complex oligosaccharide mixture that is present within milk from lactating women. Although *ca*. 20% of women lack the enzyme in their mammary glands that is responsible for the biosynthesis of 2'-FL (referred to as non-secretors), and thus, their milk is devoid of 2'-FL and other 2'-*O*-fucosylated oligosaccharides, the majority (80%) of lactating women produce milk with such a high level of 2'-FL that the substance represents the most abundant individual HMO that can be detected in pooled human milk samples. The addition of 2'-FL to term infant formulas is therefore supported on a teleological basis and is consistent with efforts to produce infant formula that closely match the nutrient composition of human milk.

The presence of HMOs in breast milk has been associated with a variety of nutritional effects including the establishment and maintenance of healthy intestinal bacterial microflora that is rich in bifidobacteria (Bode, 2009, 2012; Chichlowski *et al.*, 2011, 2012; Jantscher-Krenn and Bode, 2012; Castillo-Courtade *et al.*, 2015; Wang *et al.*, 2015; He *et al.*, 2016); reducing the adhesion of pathogens to the intestinal wall (Andersson *et al.*, 1986; Zopf and Roth, 1996; Idänpään-Heikkilä *et al.*, 1997; Newburg, 2000; Newburg *et al.*, 2005; Bode *et al.*, 2012; Hester *et al.*, 2013; Li *et al.*, 2014); modulating the maturation of intestinal enterocytes and epithelial cells (Kuntz *et al*., 2008, 2009; Holscher *et al*., 2014; Mezoff *et al*., 2016); providing nutritional support to the neonatal immune system (Newburg and Walker, 2007; Newburg, 2009), and potentially supporting the maintenance of normal cognitive, learning and memory functions of the brain (Matthies *et al.*, 1996; Murrey and Hsieh-Wilson, 2008; Mountford *et al.*, 2015; Vázquez *et al.*, 2015). For a more in-depth review please refer to the review/proceedings of the first "*International Conference on Glycobiology of Human Milk Oligosaccharides",* which was held in Copenhagen in May 2011 (Kunz *et al.*, 2012).

2'-FL is the predominant milk oligosaccharide in the milk of the FUT2 phenotype (secretor mothers) and can therefore be associated with observed health differences in infants receiving milk from secretor mothers (expressing 2'-FL into their milk) and non-secretor mothers (lacking 2'-FL in their milk). This correlation has been investigated by a number of epidemiological clinical trials in term and preterm infants and has been reported to affect the gut bifidobacterial communities in breastfed children (Lewis *et al.,* 2015), to reduce the risk for allergy (Sprenger *et al.,* 2016), mortality, sepsis and NEC (Morrow *et al.,* 2011), Crohn's disease (McGovern *et al.,* 2010) and diarrhea (Morrow *et al.,* 2004).

IV.D Metabolic Fate

Reviews of published data and information characterizing the absorption, distribution, metabolism and excretion of 2'-FL have been the subject of previous comprehensive evaluations, and this information is incorporated herein by reference to Section IV.B.4 of GRN 546 (U.S. FDA, 2014a). In brief, it is generally recognized that HMOs, including 2'-FL, are highly resistant to hydrolysis by digestive enzymes under conditions simulating the infant gastrointestinal tract (Engfer *et al*. 2000; Gnoth *et al*. 2000). Gnoth *et al*. (2001) have suggested that small quantities of 2'-FL may be transported transcellularly across the intestinal epithelium by receptor-mediated transcytosis, and/or by paracellular means, and low quantities of 2'-FL have been detected unchanged in the urine of breast-fed infants (Rudloff *et al*., 1996, 2012; Obermeier *et al.*, 1999; Chaturvedi *et al*., 2001b; Dotz *et al*., 2014). However, data from infant studies analyzing HMO digestion by intestinal bacterial microflora and HMO fecal excretion indicate that the proportion of 2'-FL that may be absorbed would be relatively small. Goehring *et al.* (2014) have reported that HMOs, including 2'-FL, are absorbed at low levels and can be detected in the urine. Concentrations in the blood and urine were 0.1 and 4% of the levels measured in milk. These results are further corroborated by the work by Marriage *et al.* (2015) demonstrating that the relative absorption[5] of 2'-FL in the plasma is in the region of 0.02 and 0.07% for infants receiving formulae supplemented with 0.2 and 1.0 g 2'-FL/L, respectively. Thus, the majority of 2'-FL consumed by infants will be transported intact to the large intestine and subjected to partial fermentation by the indigenous microbiota populations within the gastrointestinal tract (Brand-Miller *et al.*, 1995, 1998). For example, 2'-FL has been detected unchanged (by HPLC) in fecal samples of infants at levels amounting to 40 to 50% of the ingested amount following consumption of breast milk (Chaturvedi *et al.,* 2001b; Coppa *et al.*, 2001; Albrecht *et al.*, 2011).

IV.E Toxicological Studies

The safety of 2'-FL under the specified conditions of intended use in term infant formula and conventional food and beverage products is largely based on published studies characterizing the concentrations of 2'-FL in human milk (See Section IV.A), the corresponding history of safe

[5] Relative absorption was defined as the concentration of 2'-FL in plasma relative to the concentration in the feed.

consumption of 2'-FL by breast feeding infants and upon data demonstrating that crystalline 2'-FL produced by fermentation is of high purity and is qualitatively and quantitatively representative of 2'-FL that is present within human milk.

Published and unpublished toxicological studies in neonatal and mature rats and tolerance studies in neonatal piglets have been conducted, and findings from these studies further corroborate the safety of the ingredient. Comprehensive discussions of published toxicity studies as they apply to the safety of 2'-FL for use in infant formula are incorporated by reference to GRN 546 and GRN 571, and are briefly summarized in the following sections.

New studies relevant to the safety of 2'-FL, include a 90-day oral toxicity study, an *in vitro* bacterial reverse mutation assay, and an *in vitro* micronucleus assay conducted using 2'-FL produced by fermentation by Glycom as described herein. Findings from these studies are consistent with available published data characterizing the toxicity of 2'-FL and therefore provide additional evidence to corroborate safety of 2'-FL for use in infant formula. Glycom also notes that 2'-FL has been recently evaluated in a neonatal piglet model and was reported to be safe and well-tolerated when formulated in a milk replacer providing up to 291.74 mg/kg body weight/day in males and 298.99 mg/kg body weight/day in females during a 20-day treatment period (Hanlon and Thorsrud, 2014).

No studies were identified from the updated literature search to suggest that 2'-FL may be unsafe for consumption. A comparison of the test articles used in the toxicity studies is presented below in Table IV.E-1.

Table IV.E-1 Comparison of 2'-FL Test Articles Used in Toxicological Studies

Parameter	**2'-FL Specification**		
	Glycom A/S	**Glycom A/S** Coulet *et al*. (2014)	**Jennewein Biotechnologie** GRN 571; Hanlon and Thorsrud (2014)
Test Article Purity	97.6%	99%	94.1%
Manufacture Process	Fermentation	Chemical synthesis	Fermentation
Production Organism	*Escherichia coli* K-12	NA	*E. coli* BL21
Specification			
Assay by HPLC	Min. 94.0%	Min. 95.0%	≥ 90%*
D-Lactose	Max. 3.0 w/w%	Max. 3.0 w/w%	≤ 5%*
L-Fucose	Max. 1.0 w/w%	Max. 1.0 w/w%	≤ 3%*
Difucosyllactose	Max. 1.0 w/w%	Max. 1.0 w/w%	≤ 5%*
2'-Fucosyl-D–lactulose	Max. 1.0 w/w%	Max. 0.6 w/w%	NS
Fucosyl galactose	NS	NS	≤ 3%*
Protein	0.01%	0.1%	≤ 100 ppm
Ash	Max. 1.5%	0.2%	≤ 5%

2'-FL = 2'-*O*-Fucosyllactose; HPLC = high-performance liquid chromatography; NA = not applicable; NS = not specified; ppm = parts per million; w/w = weight/weight.
* Percent of total carbohydrates by HPLC (area under the curve)

IV.E.1 Repeated Dose Toxicity

The oral toxicity of 2'-FL has been evaluated in a repeat-dose 90-day subchronic toxicity study in rats conducted in accordance with the Organization for Economic Cooperation and Development (OECD) Test Guideline No. 408 (OECD, 1998a,b), with an adaptation to include the use of neonatal animals (Coulet *et al*., 2014). The 2'-FL used in the study (Glycom AS, Denmark) was produced by chemical synthesis as described in GRN 546 and had a reported purity of 99% (by HPLC, on a dry weight basis). Neonatal [post-natal day (PND 7)] Wistar [Crl:WI(Han)] pups[6] were administered 2'-FL by oral gavage at doses of 0 (water vehicle control), 2,000 (low-dose), 5,000 (mid-dose), or 6,000 (high-dose) mg/kg body weight/day from PND 7 to up to 13 weeks of age. A reference control group (15 rats/sex/group) was administered 6,000 mg/kg body weight/day of oligofructose (FOS). 2'-FL was well-tolerated at doses of up to 5,000 mg/kg body weight/day for 13 weeks, with the only notable observations reported by the authors being transient lower body weight gain[7] and colored/liquid feces during the first few days of the administration period. The authors reported three unexplained deaths, 1 male and 1 female in the 6,000 mg/kg group of 2'-FL on day 2, and 1 male in the FOS control group on day 12. Glycom notes that no cause of death could be determined following gross or histopathological investigations, and were not associated with marked changes in any other safety indices measured at the end of the study. Nevertheless, due to the unexplained deaths, a no-observed-adverse-effect level (NOAEL) of 5,000 mg/kg body weight, the mid-dose, was reported by the authors. This study (Coulet *et al*., 2014) has also been subsequently reviewed within a recent novel food opinion published by EFSA (2015a) for the use of 2'-FL in infant formula and conventional food products. That agency stated that "*Based on the decrease in the relative kidney weight in the 2'-FL high-dosed female group, two unexplained deaths in the high-dose 2'-FL group and high-dosed female group, and the significant changes in the haematological and clinical blood parameters in the 2'-FL mid- and high-dosed group, the Panel considers that the no observed adverse effect level (NOAEL) is 2 000 mg/kg body weight per day*." (EFSA, 2015a). Glycom noted that hematological effects were limited to slight reductions in (<5%) red blood cell count that were not consistent between sexes and were not associated with histopathological or gross pathological correlates. Changes in clinical chemistry parameters were limited to dose responsive reductions in AST levels[8] in both sexes. AST levels were similarly decreased by a comparable magnitude in both males and females of the FOS group (*i.e*., positive control). In the absence of further clinical chemistry, hematological or histopathological correlates, the reduction in AST levels were not considered adverse. Glycom therefore considers the NOAEL of 5,000 mg/kg body weight established by Coulet *et al*. (2014) to be appropriate.

[6] The control and high-dose groups each consisted of 15 males and 15 females, while the low- and mid-dose groups each consisted of 10 males and 10 females.
[7] No significant difference in food consumption was observed between the groups receiving 2'-FL and the control group.
[8] AST levels decreased by up to ~20% (P<0.05) in the male and female high dose 2-FL groups.

This conclusion was further corroborated by findings from other toxicity studies of 2'-FL not available to EFSA during their review. For example, study results from an oral toxicity study of 2'-FL was recently reported by Jennewein Biotechnologie within the company's GRAS notification filed by the FDA on March 20, 2015 (U.S. FDA, 2014c). The study was conducted in accordance with Good Laboratory Practice (GLP) and with consideration of OECD Test Guideline No. 408 (OECD, 1998a,b). The 2'-FL used in the study was produced by microbial fermentation and had a reported purity of 94.1% (see Table IV.D.1). A summary of the study results, as well as full study report are publically available for review in GRN 571 (Jennewein, 2015). In brief, the study was conducted using groups of 10 four-week-old male and female CD® Crl:CD Sprague-Dawley rats randomized to 1 of 2 treatment allocations administered 2'-FL in the diet at concentrations of 0 or 10%. Additional groups of 3 and 9 animals per sex were included in the control (0%) and treatment (10%) groups, respectively, and used exclusively for blood sampling. 2'-FL was well tolerated by the test animals, with the only notable effects being sporadic pale coloration of the feces in the treatment group, which was attributed to the presence of undigested 2'-FL. No animal deaths were reported. No test article-related effects in body weight, body weight gain, food consumption, water consumption, neurological parameters, hematological and blood biochemical parameters, urinalysis, ophthalmological observation, organ weights, or macroscopic or histopathological findings were observed. A NOAEL determination of 7,660 mg/kg body weight/day (corresponding to 10% dietary concentration of 2'-FL), the only dose tested, was determined by Jennewein. Glycom agrees that this NOAEL is appropriate.

IV.E.1.1 Product Specific Studies – 2'-FL Produced by Fermentation

The toxicity of 2'-FL manufactured by Glycom by fermentation, as described herein, was investigated in an adapted subchronic (90-day) oral toxicity study in 7-day-old neonatal Wistar [Crl:WI(Han)] rats (Penard, 2015). The study was modeled after the 90-day toxicity study described previously by Coulet *et al*. (2014) and was conducted in accordance with the OECD Principles of GLP. Based on the previous NOAEL determination of 5,000 mg/kg body weight for reported previously for 2'-FL by Coulet and colleagues (2014), test doses of 2,000, 4,000, and 5,000 mg 2'-FL/kg body weight/day were selected for the study. 2'-FL used in the study was produced by fermentation, had a purity of 97.6% (Glycom A/S; Lot (b) (4)), and was representative of the article of commerce. A reference group administered FOS at a dose of 5,000 mg/kg body weight/day also was included in the study. Separate recovery groups consisting of 5 males and 5 females administered the control, 2'-FL, or FOS for 90 days were terminated after a 28-day recovery period. Individual dams with reconstituted litters of at least 5 male and 5 female pups were housed in plastic cages until weaning on PND 21. All pups in each reconstituted litter were treated at the same dose level (starting on PND 7). On PND 21, pups were weaned and placed in plastic cages according to sex and dose group such that a total of 5 pups of the same sex and dose group were housed per cage. A standard diet (A04C-10) and water were provided *ad libitum*. Animals were observed twice daily for mortality

and morbidity, and clinical observations were performed daily. A detailed clinical examination was performed weekly. Body weights were assessed at time of randomization, prior to dosing, twice weekly during the first 8 weeks of the administration period, and then once weekly thereafter. Food intake also was measured twice weekly after weaning and for the first 6 weeks post-weaning, and then once weekly thereafter. Ophthalmological examinations were performed on all animals from the control, high-dose 2'-FL, and FOS groups during the last week of administration. Fasting blood and urine samples were collected from all animals of all groups for clinical pathology analysis (*i.e.*, hematology, coagulation, clinical chemistry, and urinalysis) at the end of the administration period. Clinical pathology also was performed for all animals from all groups at the end of the recovery period. Complete necropsy was performed and selected organs were removed and weighed for all animals at the end of the treatment period or at the end of the 4-week recovery period. Histopathological examinations of all organs and tissues were performed for all animals in the control, high-dose 2'-FL, and FOS groups at the end of the administration period. Kidneys from all females in the low- and mid-dose groups and in all recovery groups also were microscopically examined.

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

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

No test article-related differences in urinalysis parameters were observed between treatment groups and the water control or reference compound. No treatment-related differences in organ weights or macroscopic observations were observed between rats receiving 2'-FL and the control and reference groups. No evidence of treatment-related effects in histological observations was observed in animals receiving 2'-FL compared to control and reference groups (see Appendix A for a summary of the results of histological evaluations).

A NOAEL of 5,000 mg/kg body weight/day, the highest dose tested, was determined by the study investigators and Glycom agrees that this NOAEL is appropriate.

IV.E.2 Neonatal Piglet

The tolerability of 2'-FL produced by fermentation was evaluated in a neonatal piglet model by Hanlon and Thorsrud (2014). The study was conducted in compliance with the OECD and FDA's principles of GLP. Demonstic Yorkshire Crossbred farm piglets received commercial milk replacer diets[9] containing 0 (control), 200 (low-dose), 500 (mid-dose), or 2,000 (high-dose) mg/L of 2'-FL, corresponding to doses of 0, 29.37, 72.22, or 291.74 mg/kg body weight/day in males and 0, 29.30, 74.31, and 298.99 mg/kg body weight/day in females, respectively. Although a justification for the dose concentrations used in the study were not provided, the high-dose concentration is consistent with the intended use level of 2'-FL established by the ingredient manufacturer (Jennewein Biotechnologie GmbH) under GRN 571. The rationale for the maximum dose used in the study is unclear as infants consuming 2`-FL from human milk provided by Secretor mothers will be exposed to several-fold higher dietary intakes of 2`-FL than those provided to the piglets. The 2'-FL test article used in the study was produced by fermentation by and had a purity of 97.9% (See Table IV.D-1). Piglets were administered the liquid diet for the first 3 weeks of life[10]. Due to the imbalance of the number of male and female piglets available at the initiation of the study, animals were assigned to treatment groups strategically to ensure the control and highest dose group had an equal distribution of male and female animals. Thus, 6, 8, 7, and 6 male piglets, and 6, 4, 5, and 6 female piglets were assigned to the control, low-, mid-, and high-dose groups, respectively. Diets were offered orally *via* a feeding bowl filled by hand six times per day at a dose volume of 500 mL/kg body weight/day for at least 20 consecutive days during the study. Individual piglet doses were based on the most recent body weight measurements and food consumption was measured and recorded daily during the study. All animals were monitored for morbidity, mortality, injury, availability of food, body weight, and food efficiency during the study period. Blood samples were obtained on study days 7 and 21 for evaluation of hematological indices, blood coagulation

[9] Land O'Lakes® ProNurse® Specialty Milk Replacer from Purina Animal Nutrition, Gray Summit, Missouri.
[10] It is noted that neonatal piglets have similarities to human infants in respect to presence of specific digestive enzymes, nutrient absorption, gut closure, dietary requirements, microbial population, and gut transit time. This makes neonatal piglets a good animal model for the first 3 months of life for human infants; however, it should be noted that porcine milk contains only insignificant amounts of fucosylated oligosaccharides and do not have microflora populations adapted to metabolize these sugars (Tao *et al*. 2010; Difilippo *et al*. 2016), therefore the relevance of this animal model to study tolerance of human milk oligosaccharides is unclear.

status and blood biochemical parameters. Urine samples were collected at terminal necropsy and evaluated for urine volume, specific gravity, and pH. Organ weights were recorded for the brain, heart, kidney, large intestine, liver, small intestine, and spleen and the pH of the intestinal contents of the cecum and colon also were recorded. Histopathological examinations were performed on the organs listed above, as well as the eye, gall bladder, stomach, gross lesions, lung with bronchi, mesenteric lymph nodes, pancreas, and Peyer's patch.

Dietary administration of 2'-FL was noted by the study investigators to be "well-tolerated". All animals survived to the scheduled necropsy. Clinical observations included the following: watery feces were noted in 2 low-dose males and 2 low-dose females, 1 mid-dose male and 2 mid-dose females, and 3 high-dose males and 2 high-dose female. One high-dose male and 2 high-dose females exhibited a lack of appetite on 1 day. Lastly, 1 low-dose female exhibited a lack of appetite for 2 days during the study. These observations in appetite were not considered to be toxicologically relevant as there was no dose relationship and there were no differences in body weight observed between treated piglets and controls. No differences in food consumption were observed between treatment groups. No test-article related effects in hematological, coagulation, or blood biochemical parameters were noted. Similarly, no test article-related effects on urinalysis parameters were noted. No gross or histopathological findings were associated with the test article. Although there was a statistically significant increase in the absolute weights of the heart and kidneys for low-dose males, there was not a difference in the relative (to body weight) organ weights and thus were not considered to be test article-related.

It was thus concluded that the addition of 2'-FL to milk replacer at concentrations of up to 2,000 mg/L was well-tolerated by neonatal farm piglets and did not result in adverse health effects or impact piglet growth at doses equivalent to 291.74 mg/kg body weight/day in males and 298.99 mg/kg body weight/day in females.

IV.E.3 Other Studies Identified During Updated Literature Search

To identify new data and information relevant to the safety of 2'-FL published since the previous GRAS determinations, a comprehensive search of the published scientific literature was conducted from January 2014 through November 2015. The search was conducted using the electronic search tool, DIALOG, with several databases, including MEDLINE®, ToxFile, AGRICOLA, AGRIS, BIOSIS Toxline®, FOODLINE®: Science, Food Science and Technology Abstracts, CAB Abstracts, BIOSIS Previews®, NTIS, and EMBASE. Results of any new studies identified are summarized below.

The effects of repeat dose oral exposure to 2'-FL on synaptic plasticity and learning capabilities were investigated in adult male rats and mice (Vázquez *et al*., 2015). Briefly, 2'-FL was administered orally to C57BL/6 male mice and Sprague-Dawley male rats at dietary concentrations of 0.312% in mice for 12 weeks or 0.625% in rats for 5 weeks (targeted to

provide approximately 350 mg/kg body weight/day in both species). Control groups received the un-supplemented base diet. The 2'-FL was noted to be acquired from Inalco Pharmaceuticals (no further details provided). Mice were tested for special learning, working memory, and operant conditioning using the IntelliCage system and rats were submitted to a fixed-ratio scheduled in the Skinner box. No significant differences in daily food intake were noted between 2'-FL supplemented groups and controls in either the mice or rat study. Additionally, no differences in body weight were observed in animals receiving 2'-FL compared to controls. No other safety-related endpoints were evaluated in the study. Based on the results of the learning and memory tasks, it was concluded by the study investigators that 2'-FL-treated rodents performed significantly better than controls as measured by improved input/output curves and long-term-potentiation responses at the hippocampal CA3-CA1 synapse, improved performance of motor and cognitive tests in the IntelliCage, improved performance of rats in the Skinner box task, and increased expression of brain functional markers in rats.

In a study by Castillo-Courtade *et al*. (2015), oral administration of 1 mg of 2'-FL to ovalbumin sensitized male Balb/c mice (8- to 9-month-old) for 15 days reportedly led to reduced symptoms of food allergy.

Lastly, in a mouse model, 2'-FL feeding protected animals from clinical symptoms (weight loss) induced by aggregating-invasive-*E. coli* pathogens (He *et al.*, 2016), presumably through anti-inflammatory mechanisms.

Overall, the new studies identified in the literature did not identify any physiological or potentially toxicological effects to suggest that the use of 2'-FL may be unsafe.

IV.E.4 Genotoxicity

The genotoxicity of 2'-FL produced by chemical synthesis as previously described in GRN 546 (purity of 99%; Glycom, Denmark) was evaluated in a bacterial reverse mutation assay in *Salmonella* Typhimurium strains TA98, TA100, TA102, TA1535, and TA1537 in the presence or absence of metabolic activation (S9), using the plate-incorporation and pre-incubation methods (Coulet *et al*., 2014). This study was conducted in compliance with the OECD principles of GLP and according to OECD Test Guideline No. 471 (OECD, 1997a, 1998a). In brief, treatment with 2'-FL did not result in a biologically significant increase in the number of revertant colonies compared with the negative control at any concentration tested in the presence of absence of metabolic activation (S9).

2'-FL produced by chemical synthesis (purity of 99%; Glycom, Denmark) also was investigated in an *in vitro* mammalian cell gene mutation test conducted in L5178Y $tk^{+/-}$ mouse lymphoma cells (Coulet *et al*., 2014). The study was conducted in compliance with the OECD principles of GLP and according to OECD Test Guideline No. 476 (OECD, 1997b, 1998a). In summary, no

statistically or biologically significant increases in the frequency of mutations were observed in both the short-term and continuous experiments in which cells were treated with 2'-FL at concentration of up to 5,000 µg/mL in the presence and absence of exogenous metabolic activation (S9).

The genotoxicity of 2'-FL produced by chemical synthesis was recently evaluated in the *in vitro* mammalian cell mutation assay conducted in under GLP and in accordance with OECD Test Guideline No. 487 (Verbaan, 2015a). 2'-FL did not increase the number of micronucleated peripheral human lymphocytes at concentrations of up to 2,000 µg/mL in the presence and absence of exogenous metabolic activation (S9).

IV.E.4.1 Product Specific Studies – 2'-FL Produced by Fermentation

The mutagenicity of 2'-FL (HPLC purity = 97.6%; lot No. (b) (4) ; Glycom, Denmark) produced by fermentation, was evaluated in a bacterial reverse mutation assay in *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and in *Escherichia coli* strain WP2uvrA in the presence or absence of metabolic activation (S9), using the plate incorporation and pre-incubation methods (Verspeek-Rip, 2015). The study was conducted in accordance with the OECD principles of GLP and according to OECD Test Guideline No. 471 (OECD, 1997a, 1998a). Using the plate incorporation method, bacterial strains were treated with 2'-FL diluted in water at concentrations of 52, 164, 512, 1,600, or 5,000 µg/plate. For the pre-incubation method, bacterial strains were incubated with 2'-FL at concentrations of 492, 878, 1,568, 2,800, or 5,000 µg/plate. No cytotoxicity or precipitation was observed in any strain treated with 2'-FL in the presence or absence of S9. Treatment with 2'-FL did not result in a biologically significant increase in the number of revertant colonies compared with the negative control at any concentration in both experiments either in the presence or absence of S9. In contrast, positive control agents substantially induced an increase in the number of revertant colonies compared to the negative control. 2'-FL was determined to be non-mutagenic under the conditions of the bacterial reverse mutation assay in the presence or absence of exogenous metabolic activation at concentrations up to 5,000 µg/plate.

The genotoxicity of 2'-FL (HPLC purity = 97.6%; lot No. (b) (4) ; Glycom, Denmark) produced by fermentation was further investigated in an *in vitro* micronucleus assay conducted in cultured peripheral human lymphocytes (Verbaan, 2015b). This study was conducted in compliance with the OECD principles of GLP and according to OECD Test Guideline No. 487 (OECD, 1998a, 2014). Mitomycin C and cyclophosphamide were used as the positive controls in the absence and presence of S9, respectively, and water was used as the negative control. In the short-term exposure experiment, lymphocytes were incubated with 2'-FL at concentrations of 512, 1,600, or 2,000 µg/mL for 3 hours in the presence or absence of S9, following which the cells were rinsed and incubated for another 24 hours prior to scoring. In the long-term exposure experiment, cells were treated with 2'-FL at concentrations of 512, 1,600, or 2,000 µg/mL for

24 hours in the absence of S9. At least 1,000 binucleated cells and 1,000 mononucleated were scored for micronuclei under each treatment condition.

In both experiments, there were no signs of precipitation or cytotoxicity (as determined by the cytokinesis block proliferation index) observed in cells treated with 2'-FL at any concentration. No statistically or biologically significant increases in the frequency of mono- or bi-nucleated cells with micronuclei were observed in cells treated with 2'-FL, in both experiments. 2'-FL was determined to be non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.

IV.F Human Studies

IV.F.1 Infants

IV.F.1.1 2'-FL in Combination with GOS in Infant Formula

The safety of 2'-FL (white powdered oligosaccharide from chemical synthesis, Inalco SpA, Milan, Italy) was evaluated in a randomized, controlled, prospective study conducted in healthy, full-term, singleton infants (Marriage *et al.*, 2015). Infants were enrolled within Day of Life (DOL) 5 and were provided 1 of 3 formulae: 1) a standard, milk-based, commercially available infant formula; 2) the standard formula supplemented with 0.2 g 2'-FL/L and 2.2 g GOS/L; or 3) the standard infant formula supplemented with 1.0 g 2'-FL/L and 1.4 g GOS/L. All formulae had a caloric density of 64.3 kcal/dL (comparable to human milk) and contained a total of 2.4 g/L of non-digestible oligosaccharides[11]. The mothers of infants receiving formulae were instructed to feed the study formulae as their infant's sole source of nutrition until DOL 119. A comparator (reference) group comprised of infants consuming human milk (by breast and/or bottle) also was included. The primary endpoint was body weight gain from DOL 14 to DOL 119. Secondary endpoints included measures of tolerance and other anthropometric parameters. The presence of 2'-FL also was evaluated in blood and urine collected from a subset of infants at DOL 42 and 119 and in the comparator group at DOL 42.

A total of 338 infants completed the study; the number of infants discontinuing the study formulae was not different among the formula-fed groups. No significant differences in body weight gain, body weight during clinical visits, length, or head circumference were observed between the formulae groups and the human milk reference group. The mean daily volume of study formula consumed during the study period was similar between the control and test formula groups; however, between enrolment and DOL 28, the control group consumed significantly more formula than the group receiving the formula supplemented with 0.2 g 2'-FL (661 mL/day compared to 614 mL/day, p=0.024). The mean consumption values for other time-points and for the other formula group were not disclosed by the authors. All formulae were

[11] The standard milk-based infant formula contained 2.4 g GOS/L. The two experimental formulae contained 0.2 g 2'-FL + 2.2 g GOS per liter; and 1.0 g 2'-FL + 1.4 g GOS/L, respectively.

well-tolerated, and no significant differences in the overall percentage of infants with adverse events or serious adverse events were observed between infants receiving the experimental formulae and the standard formula. Average stool consistency, number of stools per day, and the percent of feedings associated with spit-up or vomit were comparable between all groups.

2'-FL was detected in the plasma and urine of infants provided 2'-FL in formula and in infants consuming human milk. The plasma concentrations of 2'-FL on DOL 42 reflected the amount of 2'-FL present in the feeds (*i.e*. human milk > formula containing 1.0 g 2'-FL/L > formula containing 0.2 g 2'-F/L); however, the mean plasma concentrations were not similarly correlated for samples obtained on DOL 119[12]. No 2'-FL was detected in the plasma of infants fed the un-supplemented standard milk-based commercial formula containing galactooligosaccharides (GOS). Mean urine concentrations of 2'-FL were greatest in infants fed human milk and the formula containing 1.0 g 2'-FL/L, followed by infants fed the formula containing 0.2 g 2'-FL/L and the un-supplemented standard formula. The relative excretion was calculated to be 1.44 and 1.12% for the group receiving the 0.2 g 2'-FL/L and 1.0 g 2'-FL/L formulae, respectively.

Based on the results of the study, the investigators concluded that the feeding of infant formula with a caloric density similar to that of human milk resulted in comparable growth rates to that of human milk-fed infants and that formulae supplemented with 2'-FL were well-tolerated.

IV.F.1.2 2'-FL in Combination with LNnT in Infant Formula

The safety of 2'-FL (Glycom A/S, Denmark) manufactured by chemical synthesis was evaluated in a randomized, blinded controlled multi-center parallel-design study conducted in healthy, full-term infants provided a standard term infant formula supplemented with 2'-FL (at a target concentration of 1.0 g/L reconstituted formula) in combination with LNnT (at a target concentration of 0.5 g LNnT/L reconstituted formula) from 0 to 6 months of age (Puccio *et al*., 2016). A comparator group receiving a standard intact protein infant formula without HMOs also was included. All infants received intact cow's milk protein-based follow up formula from 6 to 12 months and outcomes were measured to 12 months of age. Weight gain through 4 months was evaluated as the primary endpoint, with secondary endpoints of anthropometry (including body length, head circumference), digestive tolerance, formula compliance, and morbidity including adverse event (AE) reporting.

A total of 175 infants were enrolled in the study. The mean weight gain in the test group was non-inferior[13] to the mean weight gain in the control group. Infants receiving the test formula did not differ from control with regard to weight, length, head circumference, body mass index (BMI)

[12] This finding was hypothesized by the study investigators to be due to developmental changes in the structure and function of the gastrointestinal tract mucosa and compositional transformation of the intestinal microbiota leading to a less permeable gut and better utilization of 2'-FL by microbiota populations, respectively.
[13] Weight gain in the test group was considered "non-inferior" if the lower bound of the one-sided 97.5% confidence interval on the difference between the test and control groups was greater than the non-inferiority margin of 3 grams/day, based on the recommendations from the American Academy of Pediatrics.

or corresponding z-scores or digestive tolerance. Differences in reports of some AEs were noted in infants receiving the test formula (compared to the control); infants receiving the test formula were less likely to report bronchitis through 4 months (p=0.010), 6 months (p=0.005) and 12 months (p=0.004), and less likely to report AE clusters for lower respiratory tract infections through 12 months (p=0.027). Infants in the test group were less likely to report receiving antipyretics through 4 months (p=0.032) and antibiotics through 6 months (p=0.047) and 12 months (p=0.016). The study investigators concluded that "*Infant formula with 2'-FL and LNnT is safe, well-tolerated, and supports age-appropriate growth; it reduced the likelihood of reporting morbidity, particularly bronchitis and medication use vs control*".

Additional outcomes from this trial were evaluated including effects on early intestinal microbiota (Steenhout *et al*., 2016). Stool samples were collected at 3 months of age for assessment of microbiota using 16S rRNA gene sequencing and metagenomics, and metabolics signature was assess using proton NMR-based metabolite profiling.

The global average microbial composition for the sub-group of infants with stool samples that followed the study protocol showed similar pattern between control (n=65) and test (n=58) at the genus level, although samples obtained from infants receiving the test formula were closer to breastfed (n=34) than control samples. Calculations of microbial alpha diversity and comparison of the global microbiota composition confirmed that test was different from control at the genus level ($p<0.001$) and closer to the breastfed reference. Statistical analysis (corrected for false discovery rate) identified several taxa differentially present in control and test including *Bifidobacterium* (p=0.01), *Escherichia* (p=0.008) and unclassified *Coprobacillaceae* (p=0.01). Multivariate analysis identified several influential metabolites that discriminated between test, control and breastfed groups including phenyalanine, isoleucine, tyrosine, fecal organic acids and fucosylated compounds. The values observed for test were more similar to those observed in the breast fed group compared with control, a finding that suggests reduced protein fermentation.

The authors concluded the following: "*Together these findings indicate that the addition of 2'-FL and LNnT to a starter infant formula shifts the stool microbiota and metabolic signature towards those observed in breastfed infants*". Glycom notes that findings from this study corroborate the safety of 2'-FL and secondary measures reported in the study demonstrate that the addition of 2'-FL to infant formula is associated with nutritional outcomes that are more similar to those found in breast-fed infants.

IV.F.2 Adults

The safety and tolerability of 2'-FL was investigated in a randomized, placebo-controlled, double-blind, parallel-design study in which healthy adult volunteers (51 men and 49 women; mean age of 36.0 years) were provided 2'-FL and LNnT alone or in combination at different doses for 2 weeks (summarized in EFSA, 2015a). A comparator control group receiving

glucose as a placebo was also included. The intervention groups used in the study are summarized in Table IV.F.2-1 below. All interventions were provided as single daily bolus doses. Test articles were provided in powder form and participants were instructed to dissolve the powder in approximately 250 mL of water prior to intake in the morning with breakfast. Compliance was evaluated using a subject diary in which subjects were instructed to record each intake of the test article, which was confirmed by the collection of empty and un-opened bottles at the end of the intervention period.

Table IV.F.2-1 Interventions Used in the Two-Week Healthy Adult Study

Group No.	Daily Dose of 2'-FL (grams)	Daily Dose of LNnT (grams)
1	20	0
2	10	0
3	5	0
4	0	20
5	0	10
6	0	5
7	13.33	6.67
8	6.67	3.33
9	3.33	1.67
Control	2 grams Dextropure (glucose)	

2'-FL = 2'-*O*-fucosyllactose; LNnT = lacto-N-neotetraose.

Adverse events were monitored during the study, from first intake of study product and throughout the 2-week intervention period. Blood samples were collected at baseline and at the end of the intervention period (2 weeks) and evaluated for standard hematological and blood biochemistry parameters. Feces were collected at baseline and at the end of the intervention period (2 weeks) and evaluated for biomarkers of gastrointestinal inflammation, gut mucosal immunity, malabsorption, and dietary fiber fermentation products (including calprotectin, secretory IgA, glucose, galactose, lactose, and short-chain fatty acids). Fecal samples were also collected to assess microbiota composition at baseline, during the first week of supplementation, and at week 2. Gastrointestinal symptoms were evaluated using the Gastrointestinal Symptom Rating Scale (GSRS) and changes in bowel habits were assessed using the Bristol Stool Form Scale (BSFS).

Three subjects from each intervention group were randomly selected to participate in a bioavailability and kinetic study in which blood and urine samples were obtained. Blood was collected at pre-dose, 3, 6, and 9 hours following the intake of the study product. Urine was collected at pre-dose and once more during the day.

All adverse events reported during the study were judged to be "mild" and there were no cases of premature discontinuation from the trial due to adverse events. Most adverse events were judged to be "possibly" related to the test article; however, many symptoms were noted by the

study investigators to be common and difficult to ascertain whether they were related to the test article, to normal day-to-day variation, or to increased awareness of gastrointestinal symptoms during the trial period.

Hematological and blood biochemistry analyses obtained at the 2-week time-point remained within the normal range for all subjects and any minor changes over the course of the study compared to baseline values were not considered clinically relevant. The GSRS scores indicated that 2'-FL and LNnT were well tolerated. When compared to the placebo control, the high dose of 2'-FL (20 g) resulted in an increase in nausea, rumbling, bloating, passing gas, diarrhea, loose stools, and urgency; however, scores generally remained at a level of "mild discomfort" or less. The BSFS scores indicated a mild tendency to softer stools over the course of the study compared to baseline, but differences were small and clinically irrelevant. No statistical difference was observed between the treatment groups and the placebo controls.

The microbiota profiling results demonstrate that 2'-FL and LNnT can change the intestinal microbiota composition with the increase of *Actinobacteria* and *Bifidobacterium* being the major effect of supplementation. 2'-FL and LNnT further reduced the relative abundance of Firmicutes and Proteobacteria compared to placebo. These changes in the gut microbiota composition are considered to be favorable (Brown *et al.*, 2012). The remaining results of the study (including the bioavailability and kinetic analyses) were not available at the time of the interim report.

Overall, the interim results support that the consumption of 2'-FL and LNnT, either alone or in combination, at the doses tested, was safe and well-tolerated in healthy adult men and women. Acute intake of a bolus dose of 20 g of 2'-FL or LNnT may represent a gastro-intestinal tolerability threshold for some individuals; however, bolus exposures of 20 g of 2'-FL or LNnT are highly unlikely to be experienced by the consumer given the proposed use-levels and consequently required food intakes that would lead to such intakes.

IV.G Safety of the Production Strain

IV.G.1 Taxonomic Identity and History of Food Use

Table IV.G.1-1 Characteristics of the Host Organism *Escherichia coli* K-12 DH1

Genus	*Escherichia*
Species	*Escherichia coli*
Subspecies	not applicable
Strain	*E. coli* strain K-12 DH1
Culture collection	The German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen)
Deposition number	DSM 4235 (ATCC33849)

The taxonomic identity of the host organism *E. coli* K-12 DH1 is presented in the table above. As mentioned, *E. coli* K-12 and its derivatives (*e.g.*, DH1) are known as laboratory "safety-strains". These strains cluster together in the pan-genome comparison, and possess a smaller genome, fewer genes, and the absence of gene families that would allow them to colonize the human gut and/or produce protein-type toxins (Manning *et al.*, 1977; Smith, 1978; Bachmann, 1996; Lukjancenko *et al.*, 2010). The genomes of *E. coli* K-12 and closely related strains (*i.e.*, K and B strains) have been sequenced and compared to other strains of *E. coli*, including pathogenic strains (Blattner *et al.*, 1997; Lukjancenko *et al.*, 2010).

The construction of *E. coli* K-12 strain DH1 has been described in the literature (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996). The *E. coli* K-12 strain DH1 (*λ⁻ gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44*), was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, 2015). The DH1 strain is resistant to nalidixic acid due to the gyrA96 mutation (Hanahan, 1983). The strain can grow in minimal medium provided that it is supplemented with thiamine due to the thi-1 mutation. The recA1 mutation minimizes the recombination and increases the stability of plasmids and chromosomal DNA of the strain.

The host strain *E. coli* K-12 and its derivatives are generally recognized as safe and suitable for use as a host organism for use in the construction of modified strains used for the production of food ingredients. For example, in the EU, *E. coli* K-12 has been recommended as a safe host organism by the EU Commission and has been repeatedly assessed by EFSA as safe for the production of food and feed ingredients, additives, and food enzymes [*e.g.,* chymosin (JECFA, 2006), gamma cyclodextrin (ACNFP, 2012), L-methionine (EFSA, 2013), L-valine (EFSA, 2008), L-threonine (EFSA, 2014b), L-lysine (EFSA, 2014a), L-isoleucine (EFSA, 2010), L-tryptophan (EFSA, 2015b)]. Additionally, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has assessed *E. coli* K-12 as a safe host for food enzyme preparations [*e.g.*, chymosin (JECFA, 1991, 2006)] and the French government explicitly lists chymosin derived from *E. coli* K-12 fermentation as an approved processing aid (see www.legifrance.gouv.fr) (Legifrance, 2006). In the U.S., *E. coli* K-12 and B-strain derivatives are used in the production of a range of GRAS ingredients and food enzymes [*e.g.,* alpha cyclodextrin (U.S. FDA, 2004), chymosin (U.S. FDA, 2015f), L-leucine (U.S. FDA, 2010a,b), and β-galactosidase (U.S. FDA, 2014d)]. Comprehensive safety assessments of *E. coli* K-12 *and its derivatives* have been conducted by the U.S. Environmental Protection Agency (U.S. EPA) (U.S. EPA, 1997)[14].

Other food ingredients and/or food additives produced with *E. coli* K-12-derived strains include xylitol (Khankal *et al.*, 2008; von Rymon Lipinski, 2014), thaumatin (Daniell *et al.*, 2000; von Rymon Lipinski, 2014), tagatose (Roh *et al.*, 2000; von Rymon Lipinski, 2014), formic acid (Murarka *et al.*, 2008; Shams Yazdani and Gonzalez, 2008), L-phenylalanine (Karau and Grayson, 2014), L-tyrosine (Karau and Grayson, 2014), and L-valine (Karau and Grayson, 2014).

[14] EPA Final Safety Assessment: *http://www2.epa.gov/sites/production/files/2015-09/documents/fra004.pdf*

The production strain is removed by ultrafiltration after fermentation and before the down-stream purification sequence. Absence of the production organism from the finished ingredient (including the production strain) is verified by a number of microbiological purity criteria, including *E. coli* and *Enterobacteriaceae* and the absence of residual proteins, residual DNA, and residual bacterial endotoxins (see Section II.C.3).

IV.G.2 Safety of the Introduced Genes

IV.G.2.1 Introduction of Undesirable Traits

The use of *E. coli* K-12 and its derivatives has a long history of safe use in the production of food ingredients and drugs. The annotated genome and metabolic properties of the strain have been the subject of considerable scientific evaluation and it is well-established that *E. coli* K-12 DH1 does not exhibit inherent capacity to produce protein toxins or toxic secondary metabolites. The potential of the introduced genetic elements to alter the phenotypic properties of the source organism was considered. The metabolic capacity for synthesis of 2'-FL by *E. coli* K-12 DH1 was acquired by the use of 2 plasmid vectors (pBluescript II KS; pBBR1-MCS3). These vectors are commercially available, fully sequenced (GI:58061; GI:833818), and the identity and function of all vector genes are known. Genes inherent to the vector that are of potential safety concern include the ampicillin and tetracycline resistance genes, which are located on the pBluescript II KS and pBBR1-MCS3 vectors respectively. These genes are used exclusively as selection markers during the development of strain SCR6 and are not expressed during fermentation. Using PCR based methods, finished batches of 2'-FL have been demonstrated to be absent of detectable fragments of DNA containing the antibiotic resistance genes. Genes responsible for 2'-FL biosynthesis were selectively introduced from a verified pure culture isolate of *H. pylori* originating from a commercial culture bank or were native to *E. coli* K-12. Genes encoding the sucrose operon utilized for maintaining plasmid stability were produced synthetically (automatic oligonucleotide synthesis) from the available annotated genomes of *Klebsiella pneumonia* and *Salmonella* Typhimurium, and therefore avoid the potential introduction of undesirable genes from these pathogenic donors. The gene originating from *H. pylori* was cloned from an ATCC authenticated source and the expression vector has been fully sequenced to verify the identity of the integrated genes ensuring that the unintended insertion of extraneous genes has not occurred. In addition, successful integration of all introduced gene fragments were verified using PCR based analyses and Southern blotting techniques. All introduced genes are well characterized with respect to their function as sugar modifying enzymes, do not have homology to known protein toxins, and as enzymes involved in sugar metabolism are not reasonably be expected to introduce toxicogenic/pathogenic attributes to the host, safety concerns related to the introduced genes and corresponding gene expression products are low. In addition, finished batches of 2'-FL are absent viable counts of the production organism and have been demonstrated to be absent of detectable quantities of DNA and protein corresponding to the introduced genes (see Section II.C.4). 2'-*O*-Fucosyllactose is a high purity crystalline ingredient that is absent detectable levels of protein. As neither the

modified strain, nor the introduced genes are detectable in the finished ingredient, there is no risk of transferring genetic elements of the organism to the human commensals from consumption of 2'-FL in the diet.

IV.G.2.2 Allergenicity of Expressed Proteins

The allergenic potential of the introduced proteins has been assessed using the database and search algorithms provided by the Allergen Online tool (version 15) of the University of Nebraska (www.allergenonline.org). The database was updated last on 12th of January 2015 and contains sequences of 1897 putative and known allergens (University of Nebraska, 2015).

Allergen online and NCBI were used to compare FASTA sequences of each introduced protein to protein sequences in the databases. Allergen online searches were conducted using default settings and searches were conducted for matches to the full length sequences, matches to 80 amino acid sequence segments (sliding window) and 8-mer sequence alignments. Full length FASTA matches with *E* values of (<1e-7) and/or sequence identity greater than 50% were considered potentially cross-reactive with the aligned sequence. In accordance with Codex guidelines, FASTA also was used to search for 80 amino acid sliding window segments aligning with a match ≥35% identity to a protein in the allergen database (Codex Alimentarius Commission, 2003). FASTA and BLASTP algorithms were also used to search each protein sequence with proteins in the NCBI-Entrez database, with and without the word "allergen" as a delimiter, to identify new allergens not updated into the allergen online database and to identify potential homology to microbial proteins with a history of human exposure.

No full length sequence alignments ≥50% were identified for any inserted protein. However, a homologous alignment of 378 amino acids with an *E* value of 3.1e-19 was identified between the Gene7 sequence and *beta*-fructofuranosidase precursor protein, a minor glycoprotein allergen from *Solanum lycopersicum* (tomato plant). Analysis of the Gene7 sequence using an 80-mer sliding window identified a 46.3% identity match (*E* value = 7.0e-16) to the *beta*-fructofuranosidase precursor protein. Although a FASTA search of the Gene7 protein for 8-mer alignments did not identify any matches between Gene7 and the *beta*-fructofuranosidase precursor protein, manual searches of the 80-mer alignment identified two 6-mer matches in close proximity with one of the matches being part of a 9-mer matching alignment containing a single conservative 'substitution' of tryptophan with tyrosine. Based on the bioinformatic analyses the FASTA E score (3.1e-19) for the alignment between Gene7 and the *beta*-fructofuranosidase glycoprotein, suggests that these proteins likely share a similar structural homology. However, in general, proteins that have less than a 50% identity are unlikely to be cross-reactive (Aalberse, 2000). The significance of the 80-mer alignments at 46% identity and associated 6-mer matches within the alignment are unclear as data demonstrating that proteins sharing only 35% identity over 80 amino acids are actually cross-reactive are poor and limited to *in vitro* data suggesting specific binding at this low percent identity (Allergen Online – University of Nebraska, 2015). In addition, the results of the NCBI BLASTP analyses identified the Gene7

protein as a multi-species sucrose-6-phosphate hydrolase protein common to the *Enterobacteriaceae* genus. The Gene7 protein is part of the sucrose phosphoenolpyruvate-dependent phosphotransferase system (PTS) that is required for the utilization of sucrose by many microorganisms. A 99% identity match of the Gene7 gene to *E. coli* identified from the NCBI database is consistent with its reported origin from a *Salmonella* Typhimurium plasmid. Based on the ubiquitous presence of Gene7 in many organisms that are expected to be natural inhabitants of the human microbiome, cross-reactivity to the *beta*-fructofuranosidase precursor protein seems unlikely to occur from low-level exposure. In addition, there is no published information on the prevalence of allergy to *Solanum lycopersicum beta*-fructofuranosidase precursor glycoproteins, therefore further *in vitro* or *in vivo* tests may be impossible. Finally, Glycom also notes that the tomato is not a major food allergen.

2'-FL produced by Glycom has been demonstrated to be free of detectable protein above a detection limit of 0.0017%. 2'-FL is isolated from the fermentation medium without disruption of the cells, and as the product of Gene7 is an intracellular protein, its theoretical presence in 2'-FL would be limited to a trivial and likely undetectable fraction of the total protein that can be measured.

IV.H Expert Panel Evaluation

Glycom A/S has determined that 2'-FL produced by fermentation is GRAS for use in infant formula and in food as described in Section I.D on the basis of scientific procedures. This GRAS determination is based on data generally available in the public domain pertaining to the safety of 2'-FL, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of infant formula ingredients and food ingredients. The Expert Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. Ronald Kleinman (Mass General Hospital for Children), Dr. Robert J. Nicolosi (University of Massachusetts Lowell), and Dr. John A. Thomas (Indiana University School of Medicine).

The Expert Panel, convened by Glycom A/S, independently and critically evaluated all data and information presented herein, and also concluded that 2'-FL produced by fermentation was GRAS for use in infant formula and in food as described in Section I.D based on scientific procedures. A summary of data and information reviewed by the Expert Panel, and evaluation of such data as it pertains to the proposed GRAS uses of 2'-FL is presented in Appendix B.

IV.I Conclusion

Based on the above data and information presented herein, Glycom A/S has concluded that the intended uses of 2'-FL in infant formula and in food, as described in Section I.D, is GRAS based on scientific procedures. General recognition of Glycom A/S's GRAS determination is supported by the unanimous consensus rendered by an independent Panel of Experts, qualified by experience and scientific training, to evaluate the use of 2'-FL in infant formula and in food, who similarly concluded that the intended use of 2'-FL in infant formula and in food as described herein is GRAS.

2'-FL therefore may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21, Section 170.3 of the Code of Federal Regulations.

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Appendix A

2’-FL 13-Week Oral (Gavage) Neonatal Toxicity Study in the Rat

Summary of clinical assessments, hematological parameters, and histological evaluations

Results of Clinical Assessment

Clinical Observations - Clinical Signs by Group

AB20757 - Title.

Day numbers relative to Start Date

Sex: Male

	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Abnormal foraging					
Number of Observations	.	3	.	2	7
Number of Animals	.	3	.	2	6
Days from - to	.	70 70	.	70 70	70 84
Pedalling					
Number of Observations	.	1	.	4	3
Number of Animals	.	1	.	4	3
Days from - to	.	70 70	.	70 84	84 84
Hypersalivation					
Number of Observations	.	2	.	.	5
Number of Animals	.	2	.	.	4
Days from - to	.	28 28	.	.	21 28
Hypersalivation.					
Number of Observations	.	8	1	13	37
Number of Animals	.	6	1	6	11
Days from - to	.	70 84	70 70	70 90	35 90

Clinical Observations - Clinical Signs by Group

AB20757 - Title.

Day numbers relative to Start Date

Sex: Female

	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Abnormal foraging					
Number of Observations	.	1	.	1	3
Number of Animals	.	1	.	1	3
Days from - to	.	70 70	.	70 70	70 84
Pedalling					
Number of Observations	.	2	.	1	2
Number of Animals	.	2	.	1	2
Days from - to	.	70 70	.	84 84	70 84
Hypersalivation					
Number of Observations	.	.	.	.	3
Number of Animals	.	.	.	.	3
Days from - to	.	.	.	.	21 28
Hypersalivation.					
Number of Observations	.	6	1	7	17
Number of Animals	.	5	1	4	7
Days from - to	.	70 84	63 63	35 90	42 84

Results of Hematological Assessments

Abbreviations:
RBC: Red blood cell count
Hb: Hemoglobin
PCV: Packed cell volume
MCV: Mean corpuscular volume
MCH: Mean corpuscular hemoglobin
MCHC: Mean corpuscular hemoglobin concentration
Reti.: Reticulocyte count
Plat.: Platelet count
APTT: Activated partial thromboplastin time
Prothrom: Prothrombin time
Fib.: Fibrinogen concentration
WBC: Total white blood cell count
N: Polymorphonuclear neutrophils
L: Lymphocytes
M: Monocytes
E: Polymorphonuclear eosinophils
B: Polymorphonuclear basophils
LUC: Large unstained cells
Abs.: Absolute count

Group mean hematology parameters

AB20757

Day: 91 relative to Start Date

Group	Sex			RBC T/L	Hb g/L	PCV %	MCV fL	MCH pg	MCHC g/L	Reti.(%) %
1	m		Mean	8.833*(2)	157.0	47.12	53.34*(2)	17.79*(2)	333.5	2.67
			S.D.	0.308	5.4	1.76	1.62	0.63	6.3	0.19
			N	10	10	10	10	10	10	10
2	m	t	Mean	8.454	156.7	46.62	55.16	18.53	336.2	2.70
			S.D.	0.316	2.3	1.26	1.38	0.56	6.9	0.43
			N	9	9	9	9	9	9	9
3	m		Mean	8.542	156.8	46.24	54.18	18.38	339.3	2.60
			S.D.	0.414	4.1	1.38	1.80	1.05	9.5	0.36
			N	10	10	10	10	10	10	10
4	m		Mean	8.492	154.2	45.67	53.86	18.18	337.4	2.61
			S.D.	0.343	2.7	1.39	1.68	0.51	6.1	0.30
			N	9	9	9	9	9	9	9
5	m		Mean	8.640	157.6	47.19	54.64	18.27	334.2	2.50
			S.D.	0.414	4.4	1.54	1.23	0.70	6.3	0.25
			N	9	9	9	9	9	9	9

Statistical analysis performed - Arithmetic mean values presented.
Pairwise comparisons requested: (1) v (2), (3) v (2), (4) v (2), (5) v (2).
Statistical significances marked with a number in brackets arise from the requested pairwise comparison with that group. Others arise from automatic comparisons with the principal control.
Principal Control Group: 1

Group 1 - 0 mg/kg/day Group 2 - Ref.-5000 mg/kg/day Group 3 - 2000 mg/kg/day
Group 4 - 4000 mg/kg/day Group 5 - 5000 mg/kg/day

Group mean hematology parameters

AB20757

Day: 91 relative to Start Date

Group	Sex		Reti.abs Giga/L	Plat. Giga/L	APTT s	Prothrom s	Fibrinog Conc. g/L	WBC Giga/L
1	m	Mean	235.09	890.9	16.08	21.45	3.1000	7.556*(2)
		S.D.	18.05	79.6	0.84	1.29	0.2679	1.238
		N	10	10	10	10	10	10
2	m	t Mean	227.90	876.9	16.21	22.43	2.9305	6.394
		S.D.	32.38	101.7	1.22	2.98	0.6380	0.971
		N	9	9	10	10	10	9
3	m	Mean	221.95	880.3	16.00	21.99	3.1170	6.648
		S.D.	33.56	73.6	0.99	0.78	0.1891	1.176
		N	10	10	10	10	10	10
4	m	Mean	220.13	814.0*	16.94	23.23**	3.0938	6.966
		S.D.	21.48	50.6	1.35	1.02	0.2773	1.403
		N	9	9	9	9	9	9
5	m	Mean	216.22	815.4*	16.71	22.97**	2.9534	6.600
		S.D.	22.11	63.1	0.82	1.14	0.4168	1.192
		N	9	9	9	9	9	9

Statistical analysis performed - Arithmetic mean values presented.
Pairwise comparisons requested: (1) v (2), (3) v (2), (4) v (2), (5) v (2).
Statistical significances marked with a number in brackets arise from the requested pairwise comparison with that group. Others arise from automatic comparisons with the principal control.
Principal Control Group: 1

Group 1 - 0 mg/kg/day Group 2 - Ref.-5000 mg/kg/day Group 3 - 2000 mg/kg/day
Group 4 - 4000 mg/kg/day Group 5 - 5000 mg/kg/day

Group mean hematology parameters

AB20757

Day: 91 relative to Start Date

Group	Sex		N.Abs. Giga/L	N (%) %	L.Abs. Giga/L	L (%) %	M.Abs. Giga/L	M (%) %
1	m	Mean	1.366	18.28	5.687*(2)	75.06	0.264	3.52
		S.D.	0.246	3.17	1.111	3.79	0.059	0.82
		N	10	10	10	10	10	10
2	m	t Mean	1.228	19.03	4.773	74.84	0.211	3.30
		S.D.	0.475	6.19	0.767	6.46	0.061	0.86
		N	9	9	9	9	9	9
3	m	Mean	1.355	20.03	4.879	73.76	0.198	3.02
		S.D.	0.475	4.20	0.753	4.27	0.072	0.99
		N	10	10	10	10	10	10
4	m	Mean	1.531	21.86	5.024	72.27	0.226	3.21
		S.D.	0.450	3.51	0.985	3.77	0.071	0.69
		N	9	9	9	9	9	9
5	m	Mean	1.354	20.88	4.806	72.46	0.220	3.32
		S.D.	0.249	3.97	1.020	4.74	0.071	0.90
		N	9	9	9	9	9	9

Statistical analysis performed - Arithmetic mean values presented.
Pairwise comparisons requested: (1) v (2), (3) v (2), (4) v (2), (5) v (2).
Statistical significances marked with a number in brackets arise from the requested pairwise comparison with that group. Others arise from automatic comparisons with the principal control.
Principal Control Group: 1

Group 1 - 0 mg/kg/day Group 2 - Ref.-5000 mg/kg/day Group 3 - 2000 mg/kg/day
Group 4 - 4000 mg/kg/day Group 5 - 5000 mg/kg/day

Group mean hematology parameters

AB20757

Day: 91 relative to Start Date

Group	Sex		E.Abs. Giga/L	E (%) %	B.Abs. Giga/L	B (%) %	LUC.Abs. Giga/L	LUC (%) %
1	m	Mean	0.142	1.86	0.013	0.15	0.085*(2)	1.10
		S.D.	0.061	0.67	0.007	0.07	0.040	0.49
		N	10	10	10	10	10	10
2	m t	Mean	0.120	1.87	0.013	0.17	0.054	0.83
		S.D.	0.053	0.76	0.007	0.09	0.020	0.21
		N	9	9	9	9	9	9
3	m	Mean	0.154	2.25	0.009	0.13	0.055*	0.83
		S.D.	0.062	0.62	0.006	0.07	0.030	0.43
		N	10	10	10	10	10	10
4	m	Mean	0.119	1.69	0.013	0.19	0.057*	0.80*
		S.D.	0.040	0.45	0.009	0.08	0.027	0.38
		N	9	9	9	9	9	9
5	m	Mean	0.168	2.54	0.010	0.13	0.043**	0.66*
		S.D.	0.070	1.01	0.007	0.07	0.018	0.21
		N	9	9	9	9	9	9

Statistical analysis performed - Arithmetic mean values presented.
Pairwise comparisons requested: (1) v (2), (3) v (2), (4) v (2), (5) v (2).
Statistical significances marked with a number in brackets arise from the requested pairwise comparison with that group. Others arise from automatic comparisons with the principal control.
Principal Control Group: 1

Group 1 - 0 mg/kg/day Group 2 - Ref.-5000 mg/kg/day Group 3 - 2000 mg/kg/day
Group 4 - 4000 mg/kg/day Group 5 - 5000 mg/kg/day

Group mean hematology parameters

AB20757

Day: 92 relative to Start Date

Group	Sex		RBC T/L	Hb g/L	PCV %	MCV fL	MCH pg	MCHC g/L	Reti.(%) %
1	f	Mean	8.212**(2)	152.3**(2)	45.94**(2)	55.97	18.53	331.2	3.04
		S.D.	0.257	3.6	1.09	1.19	0.38	5.2	0.35
		N	10	10	10	10	10	10	10
2	f t	Mean	7.829	146.7	43.72	55.91	18.74	335.2	2.90
		S.D.	0.421	3.1	1.37	1.63	0.81	8.1	0.75
		N	9	9	9	9	9	9	9
3	f	Mean	7.742*	148.7	43.72**	56.47	19.22	340.6*	2.65
		S.D.	0.227	2.8	1.21	1.22	0.55	7.0	0.30
		N	10	10	10	10	10	10	10
4	f	Mean	7.976*	150.0*(2)	44.57**	55.91	18.82	336.7*	2.71
		S.D.	0.305	3.5	1.66	0.97	0.53	8.8	0.50
		N	10	10	10	10	10	10	10
5	f	Mean	7.921*	149.0	43.79**	55.29	18.83	340.5**	2.58
		S.D.	0.294	4.4	1.53	1.04	0.52	7.0	0.48
		N	10	10	10	10	10	10	10

Statistical analysis performed - Arithmetic mean values presented.
Pairwise comparisons requested: (1) v (2), (3) v (2), (4) v (2), (5) v (2).
Statistical significances marked with a number in brackets arise from the requested pairwise comparison with that group. Others arise from automatic comparisons with the principal control.
Principal Control Group: 1

Group 1 - 0 mg/kg/day Group 2 - Ref.-5000 mg/kg/day Group 3 - 2000 mg/kg/day
Group 4 - 4000 mg/kg/day Group 5 - 5000 mg/kg/day

Group mean hematology parameters

AB20757

Day: 92 relative to Start Date

Group	Sex		Reti.abs Giga/L	Plat. Giga/L	APTT s	Prothrom s	Fibrinog Conc. g/L	WBC Giga/L
1	f	Mean	247.81	792.1	14.75*(2)	22.24	2.4255	4.296*(2)
		S.D.	25.45	109.9	1.30	1.16	0.2222	0.579
		N	10	10	10	10	10	10
2	f	t Mean	225.33	834.9	15.90	22.44	2.3209	3.524
		S.D.	48.61	77.5	0.33	2.20	0.3042	0.426
		N	9	9	9	9	9	9
3	f	Mean	206.36*	798.4	15.34	23.15	2.4628	3.139**
		S.D.	24.47	75.2	1.02	1.02	0.4746	0.723
		N	10	10	10	10	10	10
4	f	Mean	215.75*	842.9	15.94*	23.73*,*(2)	2.4003	3.358**
		S.D.	39.52	57.1	0.76	0.85	0.1710	1.169
		N	10	10	9	9	9	10
5	f	Mean	204.50*	782.5	15.73*	23.72*,*(2)	2.4654	3.466**
		S.D.	40.64	119.4	0.91	0.98	0.4729	0.694
		N	10	10	9	9	9	10

Statistical analysis performed - Arithmetic mean values presented.
Pairwise comparisons requested: (1) v (2), (3) v (2), (4) v (2), (5) v (2).
Statistical significances marked with a number in brackets arise from the requested pairwise comparison with that group. Others arise from automatic comparisons with the principal control.
Principal Control Group: 1

Group 1 - 0 mg/kg/day Group 2 - Ref.-5000 mg/kg/day Group 3 - 2000 mg/kg/day
Group 4 - 4000 mg/kg/day Group 5 - 5000 mg/kg/day

Group mean hematology parameters

AB20757

Day: 92 relative to Start Date

Group	Sex		N.Abs. Giga/L	N (%) %	L.Abs. Giga/L	L (%) %	M.Abs. Giga/L	M (%) %
1	f	Mean	0.755	17.79	3.300	76.53	0.114	2.62
		S.D.	0.234	5.11	0.613	6.33	0.040	0.79
		N	10	10	10	10	10	10
2	f	t Mean	0.614	17.48	2.730	77.44	0.080	2.37
		S.D.	0.223	5.61	0.416	6.35	0.019	0.75
		N	9	9	9	9	9	9
3	f	Mean	0.598	19.81	2.371**	74.99	0.071*	2.16
		S.D.	0.172	7.26	0.557	8.43	0.037	0.90
		N	10	10	10	10	10	10
4	f	Mean	0.783	24.05*(2)	2.399**	70.82*(2)	0.088*	2.59
		S.D.	0.297	8.41	0.964	7.86	0.042	0.83
		N	10	10	10	10	10	10
5	f	Mean	0.658	19.06	2.843**	76.35	0.076*	2.11
		S.D.	0.185	5.22	0.573	6.34	0.040	0.97
		N	10	10	10	10	10	10

Statistical analysis performed - Arithmetic mean values presented.
Pairwise comparisons requested: (1) v (2), (3) v (2), (4) v (2), (5) v (2).
Statistical significances marked with a number in brackets arise from the requested pairwise comparison with that group. Others arise from automatic comparisons with the principal control.
Principal Control Group: 1

Group 1 - 0 mg/kg/day Group 2 - Ref.-5000 mg/kg/day Group 3 - 2000 mg/kg/day
Group 4 - 4000 mg/kg/day Group 5 - 5000 mg/kg/day

Group mean hematology parameters

AB20757

Day: 92 relative to Start Date

Group	Sex		E.Abs. Giga/L	E (%) %	B.Abs. Giga/L	B (%) %	LUC.Abs. Giga/L	LUC (%) %
1	f	Mean	0.093	2.24	0.005	0.12	0.032	0.76
		S.D.	0.076	1.86	0.005	0.09	0.014	0.27
		N	10	10	10	10	9	9
2	f t	Mean	0.060	1.68	0.004	0.14	0.032	0.87
		S.D.	0.025	0.61	0.005	0.09	0.012	0.41
		N	9	9	9	9	9	9
3	f	Mean	0.076	2.30	0.003	0.11	0.022	0.71
		S.D.	0.055	1.34	0.005	0.09	0.007	0.14
		N	10	10	10	10	9	9
4	f	Mean	0.062	1.79	0.004	0.10	0.024	0.68
		S.D.	0.039	0.52	0.005	0.08	0.015	0.25
		N	10	10	10	10	10	10
5	f	Mean	0.063	1.75	0.003	0.10	0.026	0.73
		S.D.	0.034	0.97	0.005	0.08	0.010	0.27
		N	10	10	10	10	9	9

Statistical analysis performed - Arithmetic mean values presented.
Pairwise comparisons requested: (1) v (2), (3) v (2), (4) v (2), (5) v (2).
Statistical significances marked with a number in brackets arise from the requested pairwise comparison with that group. Others arise from automatic comparisons with the principal control.
Principal Control Group: 1

Group 1 - 0 mg/kg/day Group 2 - Ref.-5000 mg/kg/day Group 3 - 2000 mg/kg/day
Group 4 - 4000 mg/kg/day Group 5 - 5000 mg/kg/day

Results of Histological Evaluations

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
EYES;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
Haemorrhage; retrobulbar	(0)	(0)	(0)	(0)	(0)
slight	0	0	0	0	0
OPTIC NERVES;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
BRAIN;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SPINAL CORD (CERVICAL SEGMENT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SPINAL CORD (LUMBAR SEGMENT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SPINAL CORD (THORACIC SEGMENT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
THYROID GLANDS;					
Examined	(10)	(0)	(2)	(1)	(10)
Within Normal Limits	8	0	2	1	9
Follicular hyperplasia	(2)	(0)	(0)	(0)	(0)
minimal	2	0	0	0	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
THYROID GLANDS; (continued)					
Thymic ectopia	(0)	(0)	(0)	(0)	(1)
present	0	0	0	0	1
PARATHYROID GLANDS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
PANCREAS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
THYMUS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
MANDIBULAR LYMPH NODE (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	4	0	0	0	1
Plasmacytosis	(6)	(0)	(0)	(0)	(9)
minimal	6	0	0	0	7
slight	0	0	0	0	2
MANDIBULAR GLAND (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SUBLINGUAL GLAND (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
PAROTID GLAND (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
PITUITARY GLAND;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
ADRENAL GLANDS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	9	0	0	0	9
Hypertrophy; cortical	(1)	(0)	(0)	(0)	(0)
minimal	1	0	0	0	0
Cortical vacuolation	(0)	(0)	(0)	(0)	(1)
minimal	0	0	0	0	1
SKELETAL MUSCLE (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SKIN STUDY PLAN SAMPLE;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
MAMMARY GLAND;					
Examined	(10)	(0)	(0)	(0)	(9)
Within Normal Limits	10	0	0	0	9
Not Examined: NOT PRESENT	0	0	0	0	1
STOMACH;					
Examined	(10)	(0)	(3)	(2)	(10)

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
STOMACH; (continued)					
Within Normal Limits	10	0	1	1	10
Oedema; submucosal; glandular part	(0)	(0)	(1)	(0)	(0)
minimal	0	0	1	0	0
Congestion; mucosal	(0)	(0)	(1)	(1)	(0)
minimal	0	0	1	1	0
DUODENUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
AORTA;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
ESOPHAGUS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
TRACHEA;					
Examined	(10)	(0)	(1)	(0)	(10)
Within Normal Limits	10	0	1	0	10
JEJUNUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
COLON;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
ILEUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
CECUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
MESENTERIC LYMPH NODE;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	9
Paracortex, increased	(0)	(0)	(0)	(0)	(1)
minimal	0	0	0	0	1
HEART;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	9
Inflammatory cell infiltration; subacute; myocardial; ventricular	(0)	(0)	(0)	(0)	(1)
minimal	0	0	0	0	1
LIVER;					
Examined	(10)	(0)	(1)	(0)	(10)
Within Normal Limits	2	0	0	0	1
Microgranuloma(ta)	(7)	(0)	(1)	(0)	(8)
minimal	7	0	1	0	8
Hepatocytic vacuolation; periportal	(0)	(0)	(0)	(0)	(2)
minimal	0	0	0	0	2
Necrosis; haemorrhagic; hepatocytic	(1)	(0)	(0)	(0)	(0)
slight	1	0	0	0	0
Single cell necrosis	(4)	(0)	(0)	(0)	(6)
minimal	4	0	0	0	6

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
SPLEEN;					
Examined	(10)	(0)	(0)	(1)	(10)
Within Normal Limits	10	0	0	0	10
Marginal zone (mz), increased	(0)	(0)	(0)	(1)	(0)
minimal	0	0	0	1	0
Extramedullary hematopoiesis, increased	(0)	(0)	(0)	(1)	(0)
minimal	0	0	0	1	0
LUNGS;					
Examined	(10)	(0)	(1)	(0)	(10)
Within Normal Limits	9	0	0	0	9
Metaplasia; alveolar; bone	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
Haemorrhage; alveolar	(1)	(0)	(1)	(0)	(1)
minimal	0	0	0	0	1
slight	1	0	1	0	0
BRONCHUS/BRONCHI;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
KIDNEYS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	9	0	0	0	9
Tubular dilatation; cortical	(1)	(0)	(0)	(0)	(0)
slight	1	0	0	0	0
Basophilia; tubular	(1)	(0)	(0)	(0)	(1)
minimal	1	0	0	0	1
Mineralisation; cortical	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
Nephroblastomatosis	(0)	(0)	(0)	(0)	(0)

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
KIDNEYS; (continued)					
minimal	0	0	0	0	0
Dilatation; pelvic	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
slight	0	0	0	0	0
TESTES;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
OVARIES;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
PROSTATE GLAND;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SEMINAL VESICLES;					
Examined	(10)	(0)	(1)	(0)	(10)
Within Normal Limits	10	0	0	0	10
Decreased content	(0)	(0)	(1)	(0)	(0)
minimal	0	0	1	0	0
URINARY BLADDER;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
UTERUS;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
UTERUS; (continued)					
Dilatation due to oestrus cycle	(-)	(-)	(-)	(-)	(-)
present	-	-	-	-	-
CERVIX;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
VAGINA;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
Proestrus phase of the cycle	(-)	(-)	(-)	(-)	(-)
present	-	-	-	-	-
Oestrus phase of the cycle	(-)	(-)	(-)	(-)	(-)
present	-	-	-	-	-
Metoestrus phase of the cycle	(-)	(-)	(-)	(-)	(-)
present	-	-	-	-	-
Dioestrus phase of the cycle	(-)	(-)	(-)	(-)	(-)
present	-	-	-	-	-
EPIDIDYMIDES;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
STERNUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
BONE MARROW (STERNUM);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	10	10	9	10
Number of Animals Completed:	(10)	(10)	(10)	(9)	(10)
FEMUR;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
STIFLE JOINT, FEMORO-TIBIAL, LEFT;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
HARDERIAN GLANDS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
MEDIASTINAL LYMPH NODE;					
Examined	(0)	(0)	(1)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Congestion/haemorrhage/erythrophagocytosis	(0)	(0)	(1)	(0)	(0)
moderate	0	0	1	0	0
NO CORRELATE;					
Examined	(1)	(0)	(4)	(2)	(4)
Within Normal Limits	0	0	0	0	0
No correlate	1	0	4	2	4
SCIATIC NERVE (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
EYES;					
Examined	(10)	(0)	(1)	(0)	(10)
Within Normal Limits	10	0	0	0	10
Haemorrhage; retrobulbar	(0)	(0)	(1)	(0)	(0)
slight	0	0	1	0	0
OPTIC NERVES;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
BRAIN;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SPINAL CORD (CERVICAL SEGMENT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SPINAL CORD (LUMBAR SEGMENT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SPINAL CORD (THORACIC SEGMENT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
THYROID GLANDS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	8
Follicular hyperplasia	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
THYROID GLANDS; (continued)					
Thymic ectopia	(0)	(0)	(0)	(0)	(2)
present	0	0	0	0	2
PARATHYROID GLANDS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
PANCREAS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
THYMUS;					
Examined	(10)	(0)	(0)	(1)	(10)
Within Normal Limits	10	0	0	1	10
MANDIBULAR LYMPH NODE (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	7	0	0	0	4
Plasmacytosis	(3)	(0)	(0)	(0)	(6)
minimal	3	0	0	0	6
slight	0	0	0	0	0
MANDIBULAR GLAND (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SUBLINGUAL GLAND (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
PAROTID GLAND (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
PITUITARY GLAND;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
ADRENAL GLANDS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
Hypertrophy; cortical	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
Cortical vacuolation	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
SKELETAL MUSCLE (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
SKIN STUDY PLAN SAMPLE;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
MAMMARY GLAND;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
Not Examined: NOT PRESENT	0	0	0	0	0
STOMACH;					
Examined	(10)	(1)	(0)	(1)	(10)

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
STOMACH; (continued)					
Within Normal Limits	10	1	0	1	10
Oedema; submucosal; glandular part	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
Congestion; mucosal	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
DUODENUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
AORTA;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
ESOPHAGUS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
TRACHEA;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
JEJUNUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
COLON;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
ILEUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
CECUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
MESENTERIC LYMPH NODE;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	7	0	0	0	7
Paracortex, increased	(3)	(0)	(0)	(0)	(3)
minimal	3	0	0	0	3
HEART;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
Inflammatory cell infiltration; subacute; myocardial; ventricular	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
LIVER;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	4	0	0	0	5
Microgranuloma(ta)	(4)	(0)	(0)	(0)	(4)
minimal	4	0	0	0	4
Hepatocytic vacuolation; periportal	(1)	(0)	(0)	(0)	(0)
minimal	1	0	0	0	0
Necrosis; haemorrhagic; hepatocytic	(0)	(0)	(0)	(0)	(0)
slight	0	0	0	0	0
Single cell necrosis	(2)	(0)	(0)	(0)	(1)
minimal	2	0	0	0	1

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
SPLEEN;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	9
Marginal zone (mz), increased	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
Extramedullary hematopoiesis, increased	(0)	(0)	(0)	(0)	(1)
minimal	0	0	0	0	1
LUNGS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	9
Metaplasia; alveolar; bone	(0)	(0)	(0)	(0)	(1)
minimal	0	0	0	0	1
Haemorrhage; alveolar	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
slight	0	0	0	0	0
BRONCHUS/BRONCHI;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
KIDNEYS;					
Examined	(10)	(0)	(1)	(0)	(10)
Within Normal Limits	8	0	0	0	8
Tubular dilatation; cortical	(0)	(0)	(0)	(0)	(0)
slight	0	0	0	0	0
Basophilia; tubular	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
Mineralisation; cortical	(2)	(0)	(0)	(0)	(0)
minimal	2	0	0	0	0
Nephroblastomatosis	(0)	(0)	(0)	(0)	(1)

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
KIDNEYS; (continued)					
minimal	0	0	0	0	1
Dilatation; pelvic	(0)	(0)	(1)	(0)	(1)
minimal	0	0	0	0	1
slight	0	0	1	0	0
TESTES;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
OVARIES;					
Examined	(10)	(0)	(1)	(0)	(10)
Within Normal Limits	10	0	1	0	10
PROSTATE GLAND;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
SEMINAL VESICLES;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
Decreased content	(-)	(-)	(-)	(-)	(-)
minimal	-	-	-	-	-
URINARY BLADDER;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
UTERUS;					
Examined	(10)	(2)	(1)	(2)	(10)
Within Normal Limits	5	0	0	0	8

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
UTERUS; (continued)					
Dilatation due to oestrus cycle	(5)	(2)	(1)	(2)	(2)
present	5	2	1	2	2
CERVIX;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
VAGINA;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	0	0	0	0	0
Proestrus phase of the cycle	(2)	(0)	(0)	(0)	(2)
present	2	0	0	0	2
Oestrus phase of the cycle	(5)	(0)	(0)	(0)	(4)
present	5	0	0	0	4
Metoestrus phase of the cycle	(2)	(0)	(0)	(0)	(1)
present	2	0	0	0	1
Dioestrus phase of the cycle	(1)	(0)	(0)	(0)	(3)
present	1	0	0	0	3
EPIDIDYMIDES;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
STERNUM;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
BONE MARROW (STERNUM);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Killed Terminal	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	10	9	10	10	10
Number of Animals Completed:	(10)	(9)	(10)	(10)	(10)
FEMUR;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
STIFLE JOINT, FEMORO-TIBIAL, LEFT;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
HARDERIAN GLANDS;					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10
MEDIASTINAL LYMPH NODE;					
Examined	(0)	(0)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Congestion/haemorrhage/erythrophagocytosis	(0)	(0)	(0)	(0)	(0)
moderate	0	0	0	0	0
NO CORRELATE;					
Examined	(3)	(1)	(1)	(2)	(1)
Within Normal Limits	0	0	0	0	0
No correlate	3	1	1	2	1
SCIATIC NERVE (LEFT);					
Examined	(10)	(0)	(0)	(0)	(10)
Within Normal Limits	10	0	0	0	10

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	0	0	1	0
Number of Animals Completed:	(0)	(0)	(0)	(1)	(0)
EYES;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
OPTIC NERVES;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
BRAIN;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
SPINAL CORD (CERVICAL SEGMENT);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
SPINAL CORD (LUMBAR SEGMENT);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
SPINAL CORD (THORACIC SEGMENT);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
THYROID GLANDS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
PARATHYROID GLANDS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	0	0	1	0
Number of Animals Completed:	(0)	(0)	(0)	(1)	(0)
PANCREAS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
THYMUS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
MANDIBULAR LYMPH NODE (LEFT);					
Examined	(0)	(0)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Not Examined: NOT PRESENT	0	0	0	1	0
Plasmacytosis	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
MANDIBULAR GLAND (LEFT);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
SUBLINGUAL GLAND (LEFT);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
PAROTID GLAND (LEFT);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
PITUITARY GLAND;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	0	0	1	0
Number of Animals Completed:	(0)	(0)	(0)	(1)	(0)
ADRENAL GLANDS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
SKELETAL MUSCLE (LEFT);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
SKIN STUDY PLAN SAMPLE;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
MAMMARY GLAND;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
STOMACH;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
DUODENUM;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
AORTA;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
ESOPHAGUS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	0	0	1	0
Number of Animals Completed:	(0)	(0)	(0)	(1)	(0)
TRACHEA;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
JEJUNUM;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
COLON;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
Luminal dilatation	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
ILEUM;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
Luminal dilatation	(0)	(0)	(0)	(0)	(0)
slight	0	0	0	0	0
CECUM;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	0	0
Luminal dilatation	(0)	(0)	(0)	(1)	(0)
slight	0	0	0	1	0
MESENTERIC LYMPH NODE;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	0	0	1	0
Number of Animals Completed:	(0)	(0)	(0)	(1)	(0)
HEART;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
LIVER;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	0	0
Microgranuloma(ta)	(0)	(0)	(0)	(1)	(0)
minimal	0	0	0	1	0
Congestion; sinusoidal	(0)	(0)	(0)	(1)	(0)
slight	0	0	0	1	0
SPLEEN;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	0	0
Extramedullary hematopoiesis, increased	(0)	(0)	(0)	(1)	(0)
slight	0	0	0	1	0
LUNGS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	0	0
Haemorrhage; alveolar	(0)	(0)	(0)	(1)	(0)
marked	0	0	0	1	0
Oedema; alveolar	(0)	(0)	(0)	(1)	(0)
moderate	0	0	0	1	0
BRONCHUS/BRONCHI;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	0	0	1	0
Number of Animals Completed:	(0)	(0)	(0)	(1)	(0)
KIDNEYS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
TESTES;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
OVARIES;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
PROSTATE GLAND;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
SEMINAL VESICLES;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
URINARY BLADDER;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
UTERUS;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
CERVIX;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	0	0	1	0
Number of Animals Completed:	(0)	(0)	(0)	(1)	(0)
VAGINA;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
Metoestrus phase of the cycle	(-)	(-)	(-)	(-)	(-)
present	-	-	-	-	-
EPIDIDYMIDES;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
STERNUM;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
BONE MARROW (STERNUM);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
FEMUR;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
STIFLE JOINT, FEMORO-TIBIAL, LEFT;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0
HARDERIAN GLANDS;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	MALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	0	0	1	0
Number of Animals Completed:	(0)	(0)	(0)	(1)	(0)
SKIN/SUBCUTIS;					
Examined	(0)	(0)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Scab/ulceration	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
NO CORRELATE;					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	0	0
No correlate	0	0	0	1	0
SCIATIC NERVE (LEFT);					
Examined	(0)	(0)	(0)	(1)	(0)
Within Normal Limits	0	0	0	1	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	1	0	0	0
Number of Animals Completed:	(0)	(1)	(0)	(0)	(0)
EYES;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
OPTIC NERVES;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
BRAIN;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
SPINAL CORD (CERVICAL SEGMENT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
SPINAL CORD (LUMBAR SEGMENT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
SPINAL CORD (THORACIC SEGMENT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
THYROID GLANDS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
PARATHYROID GLANDS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	1	0	0	0
Number of Animals Completed:	(0)	(1)	(0)	(0)	(0)
PANCREAS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
THYMUS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
MANDIBULAR LYMPH NODE (LEFT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Not Examined: NOT PRESENT	0	0	0	0	0
Plasmacytosis	(0)	(1)	(0)	(0)	(0)
minimal	0	1	0	0	0
MANDIBULAR GLAND (LEFT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
SUBLINGUAL GLAND (LEFT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
PAROTID GLAND (LEFT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
PITUITARY GLAND;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	1	0	0	0
Number of Animals Completed:	(0)	(1)	(0)	(0)	(0)
ADRENAL GLANDS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
SKELETAL MUSCLE (LEFT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
SKIN STUDY PLAN SAMPLE;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
MAMMARY GLAND;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
STOMACH;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
DUODENUM;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
AORTA;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
ESOPHAGUS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	1	0	0	0
Number of Animals Completed:	(0)	(1)	(0)	(0)	(0)
TRACHEA;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
JEJUNUM;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
COLON;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Luminal dilatation	(0)	(1)	(0)	(0)	(0)
minimal	0	1	0	0	0
ILEUM;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Luminal dilatation	(0)	(1)	(0)	(0)	(0)
slight	0	1	0	0	0
CECUM;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Luminal dilatation	(0)	(1)	(0)	(0)	(0)
slight	0	1	0	0	0
MESENTERIC LYMPH NODE;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0

Summary of micrcscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	1	0	0	0
Number of Animals Completed:	(0)	(1)	(0)	(0)	(0)
HEART;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
LIVER;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Microgranuloma(ta)	(0)	(0)	(0)	(0)	(0)
minimal	0	0	0	0	0
Congestion; sinusoidal	(0)	(1)	(0)	(0)	(0)
slight	0	1	0	0	0
SPLEEN;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Extramedullary hematopoiesis, increased	(0)	(1)	(0)	(0)	(0)
slight	0	1	0	0	0
LUNGS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Haemorrhage; alveolar	(0)	(1)	(0)	(0)	(0)
marked	0	1	0	0	0
Oedema; alveolar	(0)	(1)	(0)	(0)	(0)
moderate	0	1	0	0	0
BRONCHUS/BRONCHI;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	1	0	0	0
Number of Animals Completed:	(0)	(1)	(0)	(0)	(0)
KIDNEYS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
TESTES;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
OVARIES;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
PROSTATE GLAND;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
SEMINAL VESICLES;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
URINARY BLADDER;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
UTERUS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
CERVIX;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	1	0	0	0
Number of Animals Completed:	(0)	(1)	(0)	(0)	(0)
VAGINA;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Metoestrus phase of the cycle	(0)	(1)	(0)	(0)	(0)
present	0	1	0	0	0
EPIDIDYMIDES;					
Examined	(-)	(-)	(-)	(-)	(-)
Within Normal Limits	-	-	-	-	-
STERNUM;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
BONE MARROW (STERNUM);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
FEMUR;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
STIFLE JOINT, FEMORO-TIBIAL, LEFT;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0
HARDERIAN GLANDS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0

Summary of microscopic findings

AB20757

Observations: Neo-Plastic and Non Neo-Plastic	FEMALES				
Removal Reason: Found Dead	0 mg/kg/day	Ref.-5000 mg/kg/day	2000 mg/kg/day	4000 mg/kg/day	5000 mg/kg/day
Number of Animals on Study :	0	1	0	0	0
Number of Animals Completed:	(0)	(1)	(0)	(0)	(0)
SKIN/SUBCUTIS;					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
Scab/ulceration	(0)	(1)	(0)	(0)	(0)
minimal	0	1	0	0	0
NO CORRELATE;					
Examined	(0)	(0)	(0)	(0)	(0)
Within Normal Limits	0	0	0	0	0
No correlate	0	0	0	0	0
SCIATIC NERVE (LEFT);					
Examined	(0)	(1)	(0)	(0)	(0)
Within Normal Limits	0	1	0	0	0

Expert Panel Consensus Statement Concerning the Determination that 2'-*O*-Fucosyllactose (2'-FL) is Generally Recognized as Safe (GRAS) for Uses in Infant Formula and Conventional Food Products

April 8, 2016

INTRODUCTION

Glycom A/S (Glycom) convened a panel of independent scientists (the "Expert Panel"), qualified by their scientific training and relevant national and international experience in the safety evaluation of food ingredients, to conduct a critical and comprehensive assessment of the available pertinent data and information on the human-identical milk oligosaccharide 2'-*O*-fucosyllactose (2'-FL), produced by fermentation using a modified strain of *Escherichia coli* K-12 DH1, and to determine whether the intended uses of 2'-FL in term infant formula and conventional food and beverage products as described in Table A-1, would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Expert Panel consisted of the below-signed qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. Ronald Kleinman (Mass General Hospital for Children), Dr. Robert J. Nicolosi (University of Massachusetts Lowell), and Dr. John A. Thomas (Indiana University School of Medicine).

The Expert Panel, independently and collectively, critically evaluated a comprehensive package of scientific information and data compiled from literature. This information was presented in a dossier provided by Glycom [Documentation Supporting the Determination that 2'-O-Fucosyllactose (2'-FL) Produced by Fermentation is Generally Recognized as Safe (GRAS) for Use in Infant Formula and Food], which included an evaluation of all available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food uses of Glycom's 2'-FL ingredient. This information was prepared in part from a comprehensive search of the scientific literature performed by Glycom and also included information characterizing the identity and purity of the ingredient, manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, information on the history of consumption from human breast milk, and information on the safety of 2'-FL. In addition, the Expert Panel evaluated other information deemed appropriate or necessary.

Following its independent critical evaluation, and on the basis of scientific procedures, the Expert Panel unanimously concluded that 2'-FL, produced by fermentation using a modified strain of *E. coli* K-12 DH1, meeting food-grade specifications and manufactured in accordance

with current Good Manufacturing Practice (cGMP), is GRAS for use in term infant formula and conventional food products as described in Table A-1. A summary of the information reviewed by the Expert Panel is discussed.

SUMMARY AND BASIS FOR GRAS

On September 25, 2014, Glycom A/S (Glycom) submitted a notice to the United States (U.S.) Food and Drug Administration (FDA), informing the Agency of Glycom's determination that the human-identical milk oligosaccharide (HiMO) 2'-*O*-fucosyllactose (2'-FL), produced by chemical synthesis, was Generally Recognized as Safe (GRAS) for use as an ingredient in term infant formula at a maximum use level of 2,400 mg per liter, and in various conventional food and beverage products at use levels ranging from 84 to 2,400 mg per serving. The notice was filed on October 20, 2014, and designated as GRN No. 546 (Glycom A/S, 2014). Based on the information provided in the notice and on other available data, the FDA had "no questions" regarding Glycom's determination that 2'-FL is GRAS under its intended conditions of use (U.S. FDA, 2014).

Glycom has since revised the production process to also include the use of microbial fermentation for the synthesis of 2'-FL. 2'-FL produced by microbial fermentation is chemically equivalent to 2'-FL produced through chemical synthesis and is intended for use as an alternative ingredient to existing GRAS uses of 2'-FL as described in GRN 546 (Glycom A/S, 2014). 2'-FL is a naturally occurring trisaccharide that is present at high concentrations in human milk from lactating women. Based on the significant content of 2'-FL in human milk, it is often categorized as a human milk oligosaccharide (HMO). 2'-FL is a chemically defined trisaccharide that occurs only as one specific constitutional isomer comprised of L-fucose, D-galactose, and D-glucose.

The molecular structure of 2'-FL has been characterized and is documented in the literature (Kuhn *et al.*, 1956; Jenkins *et al.*, 1984; Fura and Leary, 1993; Asres and Perreault, 1996; Perreault and Costello, 1999; Ishizuka *et al.*, 1999; Rundlöf and Widmalm, 2001; Svensson *et al.*, 2002; Urashima *et al.*, 2002, 2004, 2005; Almond *et al.*, 2004; Wada *et al.*, 2008). Glycom's 2'-FL produced by fermentation is chemically and structurally identical to 2'-FL produced by Glycom using chemical synthesis methods and to 2'-FL that is present in human breast milk, as confirmed by ^{1}H- and 2D-NMR-spectroscopy. 2'-FL, therefore, has an established long history of safe consumption as a nutritive component of human breast milk in infants on the basis that the 2'-FL manufactured by Glycom is chemically identical to 2'-FL present in human breast milk.

The Expert Panel critically reviewed details of the manufacturing process for 2'-FL. The ingredient is produced by fermentation in accordance with current Good Manufacturing Practice (cGMP) and principles of Hazard Analysis and Critical Control Points (HACCP). The manufacturing process can be broadly divided into two stages.

In Stage 1 [upstream processing (USP)], D-lactose and D-sucrose are converted to 2'-FL by the cellular enzymes of the 2'-FL production microorganism, which uses D-sucrose as an exclusive energy and carbon source and D-lactose as substrate for 2'-FL biosynthesis. The production microorganism is a derivative of *Escherichia coli* K-12 DH1, which is a safe laboratory strain with a well-characterized genetic history (Hanahan, 1983; Luli and Strohl, 1990; Bachmann, 1996). *E. coli* K-12 DH1 was optimized for general oligosaccharide expression features by the introduction of several modification events related to the metabolism of various sugars, and then transformed with two plasmids carrying the required genes for 2'-FL biosynthesis. The resulting strain was designated SCR6 and has been deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Germany.

2'-FL is efficiently excreted into the fermentation broth during fermentation and the microbial biomass containing the production organism is removed from the culture supernatant by ultrafiltration/diafiltration and deactivated by heat treatment.

In Stage 2 [downstream processing (DSP)], a series of purification and isolation steps are used to generate the final high-purity 2'-FL ingredient. Quality control measures are in place during the entire purification and isolation process to ensure that final batches of 2`-FL released conform with the product specifications. The 2'-FL produced by fermentation is identical to 2'-FL present in human milk from lactating women and also to the chemically-synthesized 2'-FL described previously (GRN 546). There have been no modifications to the molecular structure of 2'-FL during its manufacture from that of the 2'-FL present in human milk.

Glycom has established food-grade specifications for 2'-FL. The specifications for 2'-FL include parameters related to physical properties, purity, water, ash content, lead, and microbiological contaminants. The specified purity of 2'-FL is set at a minimum assay value of 94.0% on a dry weight basis. Small quantities of D-lactose (Max. 3.0% w/w), L-fucose (Max. 1.0% w/w), and difucosyllactose (1.0% w/w) originating from the fermentation media also are detectable in the final high-purity ingredient. As these carbohydrates are naturally present in human breast milk, the total human milk saccharide content of the ingredient is at least 96.0%. Specifications also have been established for acetic acid, carbohydrate-type compounds, and residual proteins originating from the fermentation and downstream purification processes. All analytical methods are nationally or internationally recognized or have been validated. The Expert Panel reviewed the results from 4 batches of 2'-FL and confirms that the data demonstrate that the manufacturing process produces a consistent material in conformance with the product specifications.

The ingredient also has been evaluated for the presence of fermentation metabolites (biogenic amines, amino acids, and their metabolites), microbial endotoxins, and residual DNA, the results of which demonstrate that Glycom's 2'-FL is free from these potential contaminants. The results of batch analyses indicate that there are no detectable levels of residual proteins present in the ingredient.

The Expert Panel reviewed the data supporting the bulk stability of 2'-FL, produced by fermentation as described herein, under accelerated conditions of 60 and 80°C and ambient humidity over a 6 and 3 month storage periods, respectively. 2'-FL was stable throughout the storage periods with no measureable loss of 2-'FL or change in impurity content. The stability of the chemically synthesized 2'-FL ingredient has been evaluated in various food matrices, including a commercially-representative infant formula, yogurts, ready-to-drink flavored milk, and citrus fruit beverages (the results of which are detailed in GRN 546). As 2'-FL produced by fermentation is compositionally comparable to 2'-FL manufactured by chemical synthesis, there are no anticipated differences in their stabilities in food matrices and thus, 2'-FL has been sufficiently demonstrated to be stable in infant formula and representative food and beverages under the conditions of these studies.

2'-FL is intended for use in non-exempt term infant formula, and baby foods, including toddler formula (Table A-1). The proposed use level of 2'-FL in term infant formulas is based on providing an approximation of concentrations that have been reported for human milk samples obtained from lactating women between lactation days 5 through 100. The Expert Panel previously reviewed the estimated dietary exposures to 2'-FL among infant and toddler consumers (GRN 546). The Expert Panel noted that 2'-FL produced by fermentation will serve as an alternative to other 2'-FL sources that have previously been determined to be GRAS (*i.e.*, GRN 546, 571) and therefore will not change dietary exposures in these population groups.

2'-FL is also intended for addition to a variety of conventional food and beverage products, and stratified assessments of the dietary intake of 2'-FL by potential U.S. consumers were reviewed by the Panel during Gylcom's previous GRAS determination (*i.e.*, GRN 546). The Expert Panel understands that 2-'FL will no longer be added to baked goods and baking mixes, carbonated beverages, flavored and enhanced waters, coffee and tea, beverage whiteners, fruit flavored drinks and ades, vegetable juices and nectars, and table top sweeteners. The use levels of 2'-FL in non-dairy yogurt and yogurt also have been lowered from 10.6 g/kg to a maximum proposed use level of 5.3 g/kg. Revised intakes estimates of 2'-FL were therefore conducted using the recently published food consumption data from the U.S. National Center for Health Statistics' (NCHS) 2011-2012 National Health and Nutrition Examination Surveys (NHANES). Among the individual population groups, the highest mean and 90th percentile intakes of 2'-FL on both an absolute and per body weight basis were identified in toddlers. The mean and 90th percentile of intakes of 2'-FL in this population group were determined to be 1.12 g/person/day (equivalent to 84.9 mg/kg body weight/day), and 1.97 g/person/day (equivalent to 146.0 mg/kg body weight/day), respectively. Female adults and the elderly were observed to have the lowest mean all-user intakes of 0.49 g/person/day and female adults of childbearing age had the lowest 90th percentile intakes of 1.05 g/person/day. On a body weight basis, the lowest mean intakes of 2'-FL were identified in elderly adults at 6.8 mg/kg body weight/day, whereas female adults had the lowest 90th percentile all-user intakes at 15.3 mg/kg body weight/day.

The Expert Panel critically evaluated published data and information characterizing the safety of 2'-FL. 2'-FL is one of the naturally occurring fucosylated milk oligosaccharides present in some mammalian milks (Urashima *et al.*, 2001; Castanys-Muñoz *et al.*, 2013), with the highest concentrations of 2'-FL occurring in milk from lactating women (Kuhn *et al.*, 1955). 2'-FL therefore has an established history of safe consumption by infants consuming human milk.

As discussed previously in GRN 546, the 2'-FL content of human milk has been reported in several publications from independent research groups, whereby extensive data have been provided according to secretor and Lewis-blood group status (Thurl *et al.*, 1996, 2010; Coppa *et al.*, 2011; Galeotti *et al.*, 2012, 2014), ethnicity (Erney *et al.*, 2000; Musumeci *et al.*, 2006), lactation period (Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Asakuma *et al.*, 2008; Leo *et al.*, 2010; Gabrielli *et al.*, 2011; Bao *et al.*, 2013), term/preterm birth (Nakhla *et al.*, 1999; Gabrielli *et al.*, 2011), and other studies measuring the content of mature milk (Chaturvedi *et al.*, 1997, 2001a; Nakhla *et al.*, 1999; Erney *et al.*, 2000, 2001; Sumiyoshi *et al.*, 2003; Morrow *et al.*, 2004; Leo *et al.*, 2010; Thurl *et al.*, 2010; Asakuma *et al.*, 2011; Coppa *et al.*, 2011; Galeotti *et al.*, 2012; Smilowitz *et al.*, 2013; Hong *et al.*, 2014; Balogh *et al.*, 2015). Based on Glycom's comprehensive review of the literature, a use level of 2,400 mg/L was considered representative of mean concentrations that have been reliably measured in human milk samples from full term birth mothers across a variety of demographic groups, Lewis body genotypes, and lactational stages (GRN 546). Following a similar review of the published literature, Jennewein Biotechnologie reported mean concentrations of *"~2.6 g/L"* for 2'-FL in human milk samples and established an arbitrary use level of 2 g/L for 2'-FL for term infant formula to approximate levels occurring in human milk (GRN 571).

Reviews of published data and information characterizing the absorption, distribution, metabolism and excretion of 2'-FL have been the subject of previous comprehensive evaluations, and this information is incorporated herein by reference to GRN 546. In brief, it is generally recognized that HMOs, including 2'-FL, are highly resistant to hydrolysis by digestive enzymes under conditions simulating the infant gastrointestinal tract (Engfer *et al*. 2000; Gnoth *et al*. 2000). Small quantities of 2'-FL may be transported transcellularly across the intestinal epithelium by receptor-mediated transcytosis, and/or by paracellular means (Gnoth *et al.*, 2001; Rudloff *et al*., 1996, 2012; Obermeier *et al.*, 1999; Chaturvedi *et al*., 2001b; Dotz *et al*., 2014); however, data from infant studies indicate that the proportion of 2'-FL that may be absorbed would be relatively small. The majority of 2'-FL consumed by infants is expected to be transported intact to the large intestine and subjected to partial fermentation by the indigenous microbiota populations within the gastrointestinal tract but largely excreted unchanged in the feces (Brand-Miller *et al.*, 1995, 1998; Chaturvedi *et al.,* 2001b; Coppa *et al.*, 2001; Albrecht *et al.*, 2011).

Comprehensive discussions of the published toxicity studies as they apply to the safety of 2'-FL for use in infant formula were incorporated by reference to GRN 546 and GRN 571. The Expert

Panel critically evaluated the new studies relevant to the safety of 2'-FL, including, among others, the results of a product-specific 90-day oral toxicity study, an *in vitro* bacterial reverse mutation assay, and an *in vitro* micronucleus assay commissioned by Glycom as described herein. No new studies have been identified since the previous GRAS determinations to suggest that 2'-FL may be unsafe.

The Expert Panel has reviewed the results of a published 90-day study in rats conducted in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline No. 408 (OECD, 1998a,b), with an adaptation to include the use of neonatal rats (Coulet *et al*., 2014)[1]. Neonatal (post-natal day 7) Wistar [Crl:WI(Han)] pups[2] were administered 2'-FL by oral gavage at doses of 0 (water vehicle control), 2,000 (low-dose), 5,000 (mid-dose), or 6,000 (high-dose) mg/kg body weight/day from post-natal day 7 to up to 13 weeks of age. A reference control group (15 rats/sex/group) was administered 6,000 mg/kg body weight/day of oligofructose (FOS). 2'-FL was well-tolerated at doses of up to 5,000 mg/kg body weight/day for 13 weeks, with the only notable observations reported by the authors being transient lower body weight gain and colored/liquid feces during the first few days of the administration period. The authors reported three unexplained deaths, 1 male and 1 female in the 6,000 mg/kg group on day 2, and one male in the FOS control group on day 12. Glycom notes that no cause of death could be determined following gross or histopathological investigations, and the deaths were not associated with marked changes in any other safety indices measured at the end of the study. Nevertheless, due to the unexplained deaths, a conservative NOAEL of 5,000 mg/kg body weight, the mid-dose, was established by the authors. The Coulet study has also been subsequently reviewed within a recent novel food opinion published by the European Food Safety Authority (EFSA, 2015) for the use of 2'-FL in infant formula and conventional food products. The agency stated that "*Based on the decrease in the relative kidney weight in the 2'-FL high-dosed female group, two unexplained deaths in the high-dose 2'-FL group and high-dosed female group, and the significant changes in the haematological and clinical blood parameters in the 2'-FL mid- and high-dosed group, the Panel considers that the no observed adverse effect level (NOAEL) is 2,000 mg/kg body weight per day*." (EFSA, 2015). The Expert Panel noted that the changes in hematological and clinical blood parameters (*i.e*., RBC and AST) noted by EFSA in the mid-dose group were slight (*i.e*., <5%), not consistent between sexes and/or not associated with other clinical chemistry, hematological or histopathological correlates. It is the opinion of the Expert Panel that the NOAEL of 5,000 mg/kg body weight/day as originally established by the study authors was appropriate. This conclusion is corroborated by the results of additional toxicity studies of 2'-FL not available to EFSA during their review. These studies are discussed further below.

[1] The 2'-FL used in the study (Glycom AS, Denmark) was produced by chemical synthesis as described in GRN 546 and had a purity of 99% (by HPLC, on a dry weight basis).
[2] The control and high-dose groups each consisted of 15 males and 15 females, while the low- and mid-dose groups each consisted of 10 males and 10 females.

The toxicity of a 2'-FL preparation manufactured by Jennewein Biotechnologie using microbial fermentation was evaluated in a subchronic single dose oral toxicity study in Sprague-Dawley rats. The full study report is publically disclosed in Jennewein's GRAS notification to the FDA (GRN 571). In brief, the study was conducted in consideration of OECD Guideline 408 using groups of ten 4-week-old CD® Crl:CD Sprague-Dawley rats administered 2'-FL (purity of 94.1%) in the diet at concentrations of 0 or 10%. No premature deaths were observed and no test article-related effects in clinical signs, external appearance, feces, body weight, body weight gain, food consumption, water consumption, neurological parameters, hematological and blood biochemical parameters, urinalysis, ophthalmological observation, organ weights, or macroscopic or histopathological findings were observed. A NOAEL determination of 7,660 mg/kg body weight/day, corresponding to 10% dietary concentration of 2'-FL, was reported by Jennewein based on the results of the study.

The toxicity of 2'-FL, manufactured by Glycom using fermentation as described herein, was evaluated in a 90-day oral toxicity study (Penard, 2015). The study was conducted using a slight modification of OECD 408 guidelines to utilize neonatal rats and used the same study design reported in a previous study by Coulet *et al.* (2014). Seven-day-old Wistar [Crl:WI(Han)] rats were administered 2'-FL at doses of 0 (control), 2,000, 4,000, or 5,000 mg 2'-FL/kg body weight/day or the reference compound, FOS at a dose of 5,000 mg/kg body weight/day by gavage for 90 or 91 days. No test article-related mortalities occurred during the study. The majority of animals receiving the reference compound presented with liquid feces, which was also observed in mid- and high-dose animals receiving 2'-FL. Mid- and high-dose animals receiving 2'-FL also had soiled urogenital regions. Hypersalivation, abnormal foraging and/or pedaling were observed in animals receiving the reference compound and also in the mid- and high-dose groups receiving 2'-FL from day 35 onward, with the incidence of these clinical signs being most prominent in the high-dose 2'-FL group. No test article-related ophthalmological findings were observed. No remarkable effects in body weight, body weight gain, or food consumption were observed. No toxicologically relevant effects in developmental, learning, motor, or behavioral markers were observed at any dose level. Minor differences in certain hematological parameters were deemed to be of no toxicological significance and changes in serum chemistry parameters were small in magnitude and/or within the normal historical control data range and were considered to be non-adverse. A NOAEL of 5,000 mg/kg body weight/day, the highest dose tested, was determined for this study. A copy of the full study report was made available to the Expert Panel and following a critical review of all information presented in the study report the Expert Panel similarly concluded that the authors' NOAEL determination of 5,000 mg/kg body weight was appropriate.

The tolerability of 2'-FL produced by fermentation was also evaluated in a neonatal piglet model. The study was conducted in compliance with principles of GLP (Hanlon and Thorsrud, 2014).

Demonstic Yorkshire Crossbred farm piglets received liquid diets[3] containing 0 (control), 200 (low-dose), 500 (mid-dose), or 2,000 (high-dose) mg/L of 2'-FL[4], corresponding to doses of 0, 29.37, 72.22, or 291.74 mg/kg body weight/day in males and 0, 29.30, 74.31, and 298.99 mg/kg body weight/day in females, respectively. Piglets were administered the liquid diet for the first 3 weeks of life (modelling the first three weeks of human life). Diets were offered orally *via* a feeding bowl filled by hand 6 times per day at a dose volume of 500 mL/kg body weight/day for at least 20 consecutive days during the study. Dietary administration of 2'-FL was noted by the study investigators to be well-tolerated. All animals survived to the scheduled necropsy. Clinical observations included the following: watery feces were noted in 2 low-dose males and 2 low-dose females, 1 mid-dose male and 2 mid-dose females, and 3 high-dose males and 2 high-dose females. Noted observations of lack of appetite in certain animals were not considered to be toxicologically relevant as there was no dose relationship and there were no differences in body weight observed between treated piglets and controls. No differences in food consumption were observed between treatment groups. No test-article related effects in hematological, coagulation, or blood biochemical parameters were noted. Similarly, no test article-related effects on urinalysis parameters were noted. No gross or histopathological findings were associated with the test article. Although there was a statistically significant increase in the absolute weights of the heart and kidneys for low-dose males, there was not a difference in the relative (to body weight) organ weights and thus were not considered to be test article-related.

It was thus concluded that the addition of 2'-FL to milk replacer at concentrations of up to 2,000 mg/L was well-tolerated by neonatal farm piglets and did not result in adverse health effects or impact piglet growth at doses equivalent to 291.74 mg/kg body weight/day in males and 298.99 mg/kg body weight/day in females.

The toxicological dataset also included the results of a bacterial reverse mutation assay and an *in vitro* mammalian cell gene mutation test in L5178Y tk+/- mouse lymphoma cells conducted with the chemically-synthesized 2'-FL ingredient (Coulet *et al*., 2014). Both tests were conducted in compliance with the OECD principles of GLP and according to OECD Testing Guidelines No. 471 and 476, respectively. All test results reported indicate that 2'-FL is non-mutagenic (OECD, 1997a,b). These results were corroborated by the results of product-specific genotoxicity studies conducted using 2'-FL produced by fermentation as described herein and included a bacterial reverse mutation assay (Verspeek-Rip, 2015) and an *in vitro* micronucleus assay conducted in cultured peripheral human lymphocytes (Verbaan, 2015b). The full study reports of these studies were made available to the Expert Panel and findings from these studies demonstrate that 2'-FL is not genotoxic.

[3] Diets were based on commercially available specialty milk replacer from Purina Animal Nutrition.
[4] The 2'-FL was produced by fermentation as described within GRN 571 and was characterized as the following: 97.9% 2'-FL, 4.2% water, 0.37% ash, 3.3% difucosyllactose, 1.9% fucosyl-galactose, and <50 ppm protein (Jennewein, 2015).

Other studies identified from an updated literature search also were reviewed by the Expert Panel, including the results of a repeat dose rat and mouse study evaluating synaptic plasticity and learning following oral exposure to 2'-FL (Vázquez *et al*., 2015) and a 15-day mouse study examining the effect of oral administration of 2'-FL on symptoms of food allergy (Castillo-Courtade *et al*., 2015). Overall, results from the new studies identified in the literature did not indicate any physiological or potentially toxicological effects that suggest the use of 2'-FL may be unsafe.

The Expert Panel reviewed published studies evaluating the effects of 2'-FL consumption in healthy term infants. The safety of 2'-FL (Inalco SpA, Milan, Italy) was evaluated in a randomized, controlled, prospective study conducted in healthy, full-term, singleton infants (Marriage *et al.*, 2015). Infants were enrolled within Day of Life (DOL) 5 and were provided one of three formulae - a standard, milk-based, commercially available infant formula, the standard formula supplemented with 0.2 g 2'-FL/L and 2.2 g GOS/L, or the standard infant formula supplemented with 1.0 g 2'-FL/L and 1.4 g GOS/L. All formulae had a caloric density of 64.3 kcal/dL (comparable to human milk) and contained a total of 2.4 g/L of non-digestible oligosaccharides. The mothers of infants receiving formulae were instructed to feed the study formulae as their infant's sole source of nutrition until DOL 119. A comparator (reference) group comprised of infants consuming human milk (by breast and/or bottle) also was included. A total of 338 infants completed the study; the number of infants discontinuing the study formulae was not different among the formula-fed groups. No significant differences in body weight gain, body weight, length, or head circumference were observed between the formulae groups and the human milk reference group. All formulae were well-tolerated, and no significant differences in the overall percentage of infants with adverse events or serious adverse events were observed between infants receiving the experimental formulae and the standard formula. Average stool consistency, number of stools per day, and the percent of feedings associated with spit-up or vomit were comparable between all groups. 2'-FL was detected in the plasma and urine of infants provided 2'-FL in formula and in infants consuming human milk; plasma concentrations of 2'-FL were correlated with dietary concentrations of 2'-FL for samples obtained on DOL 42 but was not similarly correlated for samples obtained at DOL 119[5]. No 2'-FL was detected in the plasma of infants fed the un-supplemented standard milk-based commercial formula containing GOS. Based on the results of the study, the investigators concluded that the feeding of infant formula with a caloric density similar to that of human milk resulted in comparable growth rates to that of human milk-fed infants and that formulae supplemented with 2'-FL were well-tolerated.

[5] This finding was hypothesized by the study investigators to be due to developmental changes in the structure and function of the gastrointestinal tract mucosa and compositional transformation of the intestinal microbiota leading to a less permeable gut and better utilization of 2'-FL by microbiota populations, respectively.

The Expert Panel was provided abstracts detailing interim findings from a safety study of 2'-FL[6] conducted in healthy term infants (n=175) 0 to 14 days of age born to mothers residing in Italy and Belgium [ClinicalTrials.gov NCT01715246]. The study was a randomized, blinded, controlled, multi-center, parallel-design study conducted in healthy, full-term infants provided a standard term infant formula supplemented with 2'-FL (at a target concentration of 1.0 g/L reconstituted formula) in combination with lacto-N-neotetraose (LNnT; at a target concentration of 0.5 g LNnT/L reconstituted formula) from 0 to 6 months of age (Puccio *et al*., 2016). Infants randomized to the control group received a standard intact protein infant formula without HMOs. All infants received intact cow's milk protein-based follow up formula from 6 to 12 months and subjects were followed to 12 months of age. Weight gain through 4 months was evaluated as the primary endpoint, with secondary endpoints being anthropometry including body length, head circumference, digestive tolerance, formula compliance, and morbidity including adverse event (AE) reporting. The mean weight gain in the test group was non-inferior[7] to the mean weight gain in the control group. Infants receiving the test formula did not differ from control with regard to weight, length, head circumference, body mass index (BMI) or corresponding z-scores or digestive tolerance. Infants receiving the test formula were less likely to report bronchitis through 4 (p=0.010), 6 (p= 0.005) and 12 months (p=0.004), and less likely to report AE clusters for lower respiratory tract infections through 12 months (p=0.027). Infants in the test group were less likely to report receiving antipyretics through 4 months (p=0.032) and antibiotics through 6 (p=0.047) and 12 months (p=0.016). The study investigators concluded that "*Infant formula with 2'-FL and LNnT is safe, well-tolerated, and supports age-appropriate growth; it reduced the likelihood of reporting morbidity, particularly bronchitis and medication use vs control*"

Additional secondary measures of 2'-FL on fecal microbiota composition were reported by Steenhout *et al*., (2016) for a sub-group of infants for which stool samples were collected at 3 months of age. Microbiota composition was evaluated using 16S rRNA gene sequencing and metagenomics, and metabolics signatures were assessed using proton NMR-based profiling. The global average microbial composition for the sub-group of infants with stool samples displayed a similar pattern between the control (n=65) and test (n=58) groups at the genus level, although samples obtained from infants receiving the test formula were closer to breastfed (n=34) than control samples. Calculations of microbial alpha diversity and comparison of the global microbiota composition confirmed that test was different from control at the genus level ($p<0.001$) and closer to the breastfed reference. Statistical analysis (corrected for false discovery rate) identified several taxa differentially present in control and test including *Bifidobacterium* (p=0.01), *Escherichia* (p=0.008) and unclassified *Coprobacillaceae* (p=0.01).

[6] 2'-FL used in the study was produced by Glycom A/S using chemical synthesis procedures as described in GRN 546.

[7] Weight gain in the test group was considered "non-inferior" if the lower bound of the one-sided 97.5% confidence interval on the difference between the test and control groups was greater than the non-inferiority margin of 3 grams/day, based on the recommendations from the American Academy of Pediatrics.

Multivariate analysis identified several influential metabolites that discriminated between test, control and breastfed groups including phenylalanine, isoleucine, tyrosine, fecal organic acids and fucosylated compounds. The values observed for test were more similar to those observed in the breast fed group compared with control, a finding that suggests reduced protein fermentation. The authors concluded: "*Together these findings indicate that the addition of 2'-FL and LNnT to a starter infant formula shifts the stool microbiota and metabolic signature towards those observed in breastfed infants*". The Expert Panel concluded that preliminary findings reported by Puccio *et al*., (2015) and Steenhout *et al*., (2016) provide corroborative evidence to support the safety and suitability of 2'-FL for use in infant formula.

The safety and tolerance of 2'-FL was investigated in a randomized, placebo controlled, double-blind, parallel-designed study in which healthy adult volunteers (51 men and 49 women; mean age of 36.0 years) were provided 2'-FL and LNnT alone or in combination at different doses for two weeks (summarized in EFSA, 2015). 2'-FL and LNnT were provided at bolus daily doses of 5, 10, or 20 g/day. A comparator group receiving 2 g glucose as a placebo also was included. Interim results for this study available at the time of the GRAS determination were as follows: adverse events reported during the study were judged to be "mild" and "possibly" related to the test article[8]. All subjects enrolled completed the study (there were no withdrawals). Bolus doses of 20 g 2'-FL or LNnT may represent a gastro-intestinal tolerability threshold for some individuals; however, it is noted that bolus exposures of 20 g of these compounds are unlikely to be experienced by a consumer given the proposed use-levels of 2'-FL and consequently required food intakes that would lead to such exposures. For example, the highest estimated dietary intakes of 2'-FL among heavy consumers of foods to which 2'-FL may be added were estimated to be approximately 1.97 g/person/day (identified in the toddler population), based on the exposure analyses described herein using the most recent consumption data from the 2011-2012 NHANES dataset (USDA, 2014; CDC, 2015). Hematological and blood biochemistry analyses obtained at the 2-week time-point remained within the normal range for all subjects and any minor changes over the course of the study compared to baseline values were not considered clinically relevant. Scores from the Gastrointestinal Symptom Rating Scale (GSRS) indicated that the compounds were well-tolerated and results from the Bristol Stool Form Scale indicated a mild tendency to softer stools over the course of the study compared to baseline, but differences were small and clinically irrelevant. The interim results support that the consumption of 2'-FL (alone and in combination with LNnT) is safe in healthy adult men and women. A copy of the full study report was made available to the Expert Panel within Glycom's GRAS documentation and the Expert Panel considered this information to be corroborative of safety. There were no findings reported by the authors to suggest that 2'-FL would be unsafe under the conditions of intended use described herein.

[8] It was noted by the study investigators that many symptoms were common and difficult to ascertain whether they were related to the test article, to normal day-to-day variation, or to increased awareness of gastrointestinal symptoms during the trial period.

The potential allergenicity of 2'-FL was considered during Glycom's GRAS determination. Specifically, the introduction of raw materials of allergenic potential originating from the fermentation and downstream processing steps were considered. A bioinformatic assessment of potential cross-reactivity of the introduced expression proteins in the fermentation organism also was conducted. Glycom noted that lactose is used as a raw material during the fermentation process and therefore in accordance with FALCPA, labeling of the bulk ingredient 'contains milk' would be required. No other allergenic sources are used during manufacturing. The bioinformatic evaluation of the introduced proteins expressed by the production organism was conducted using the database and search algorithms provided by the Allergen Online tool (version 15) of the University of Nebraska as well as the use of FASTA and BLASTP algorisms to search each protein sequence with proteins in the NCBI Entrez database. No full length FASTA matches with E-values of (<1e-7) and/or sequence identity greater than 50% were identified. As proteins that have less than 50% identity with an allergen are unlikely to be cross-reactive, the introduced proteins were considered to be of low allergenic potential (Aalberse, 2000). In addition to the full length searches, FASTA also was used to search for 80 amino acid sliding window segments aligning with a match of ≥35% identity to a protein in the allergen database (Codex Alimentarius Commission, 2003). A homologous alignment of 378 amino acids with an *E* value of 3.1e-19 was identified between the introduced sucrose-6-phosphate hydrolase sequence and *beta*-fructofuranosidase precursor protein, a minor glycoprotein allergen from *Solanum lycopersicum* (tomato plant). Further analyses using NCBI BLASTP databases identified the protein as a multi-species sucrose-6-phosphate hydrolase protein common to the *Enterobacteriaceae* genus. Based on the ubiquitous presence of this hydrolase in many organisms that are expected to be natural inhabitants of the human microbiome, cross-reactivity to the *beta*-fructofuranosidase precursor protein seems unlikely to occur from low-level exposure. Furthermore, 2'-FL produced by Glycom has been demonstrated to be free of protein above a detection limit of 0.0017%. 2'-FL is isolated from the fermentation medium without disruption of the cells, and as sucrose-6-phosphate hydrolase is an intracellular protein, its theoretical presence in 2'-FL would be limited to a trivial and likely undetectable fraction of the total protein that can be measured. Based on the purification processed utilized during the manufacturing process and absence of detectable protein in the ingredient, the Expert Panel considered the risk of allergenicity to be very low.

Overall, the available preclinical and clinical studies, in conjunction with data and information characterizing the content of 2'-FL in milk samples from lactating women, were considered by the Expert Panel to support a determination that the addition of high purity 2'-FL, produced by Glycom *via* chemical synthesis or microbial fermentation, to term infant formula at a use level of up to 2.4 g/L and specified conventional food and beverage products at a use levels indicated in Table A-1, is GRAS by scientific procedures.

CONCLUSION

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that 2'-*O*-fucosyllactose (2'-FL), produced by fermentation using a modified strain of *E. coli* K-12 DH1, meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice, is safe and suitable for use as an ingredient in non-exempt term infant formula and specified conventional food and beverage products described in Table A-1.

We, the members of the Expert Panel, have independently and collectively critically evaluated the information summarized above and conclude that 2'-*O*-fucosyllactose (2'-FL), produced by fermentation using a modified strain of *E. coli* K-12 DH1, meeting appropriate food-grade specifications and manufactured consistent with current Good Manufacturing Practice is GRAS, on the basis of scientific procedures, for use in term infant formula and specified conventional food and beverage products as described in Table A-1.

It is our opinion that other qualified experts would concur with these conclusions.

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Attachment A1

Intended Food Uses and Use Levels for 2'-FL in the United States

Table A-1 Intended Food Uses and Use Levels for 2'-FL in the United States				
Food Category	**Proposed Food-Uses**	**RACC[a]**	**Proposed Use Level (g/RACC)**	**Proposed Maximum Use Level (g/kg or g/L)[b]**
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	1.2	5
	Sports, Isotonic, and Energy Drinks	240 mL	0.28	1.2
Dairy Product Analogs	Imitation Milks	240 mL	0.28	1.2
	Non-Dairy Yogurt	225 g	1.2	5.3
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.24	2.4
	Toddler Formulas	100 mL[c]	0.24	2.4
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
Milk, Whole and Skim	Unflavored Pasteurized and Sterilized milk[d]	240 mL	0.28	1.2
Milk Products	Buttermilk	240 mL	0.28	1.2
	Flavored Milk	240 mL	0.28	1.2
	Milk-Based Meal Replacement Drinks, for Weight Reduction	240 mL	1.2	5
	Yogurt	225 g	1.2	5.3
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.28	1.2

2'-FL = 2'-*O*-fucosyllactose; RACC = Reference Amounts Customarily Consumed.
[a] Serving sizes were based on RACCs per Eating Occasion in the United States Code of Federal Regulations (21 CFR §101.12 - U.S. FDA, 2015c).
[b] The proposed maximum use level is presented on a g/kg basis for solids and on a g/L basis for liquids.
[c] RACC not available, 100 mL employed as an approximation.
[d] Milk is a standardized food in the United States. When the milk is fortified with 2'-FL, it will then be classified as a milk product. The intake of the category "unflavored pasteurized and sterilized milks" was used here as a conservative proxy for the dietary pattern of the fortified milk drink product.

Pages 000114-003064 have been removed in accordance with copyright laws. The list of references that have been removed are on pages 000053-000067 and pages 000105-000111.

SUBMISSION END