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Dec 27th, 2021

Dr. Paulette Gaynor
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

Dear Dr. Gaynor:

Re: GRAS Notice for 2'-Fucosyllactose

In accordance with 21 CFR §170 Subpart E consisting of §§ 170.203 through 170.285, Kyowa Hakko Bio Co., Ltd. (Kyowa; 1-9-2, Otemachi, Chiyoda-ku, Tokyo, 100-0004, Japan), as the notifier, is submitting one hard copy and one electronic copy (on CD) of all data and information supporting the company's conclusion that 2'-fucosyllactose (2'-FL) is GRAS on the basis of scientific procedures, for use in non-exempt term infant formula and various conventional food and beverage products across multiple food categories; these food uses of 2'-FL are therefore not subject to the premarket approval requirements of the *Federal Food, Drug and Cosmetic Act*. Information setting forth the basis for Kyowa's GRAS conclusion, as well as a consensus opinion of an independent panel of experts, 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 12.1.5.

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,

[redacted]

External Relations Department, Manager
Kyowa Hakko Bio Co., Ltd.

GRAS NOTICE FOR 2'-FUCOSYLLACTOSE FOR USE IN NON-EXEMPT INFANT FORMULA AND SPECIFIED CONVENTIONAL FOOD PRODUCTS

SUBMITTED TO:
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5001 Campus Drive
College Park, MD
20740 USA

SUBMITTED BY:
Kyowa Hakko Bio Co., Ltd.
1-9-2, Otemachi, Choyoda-ku
Tokyo, 100-0004,
Japan

DATE:
27 December 2021

GRAS Notice for 2'-Fucosyllactose for Use in Non-Exempt Infant Formula and Specified Conventional Food Products

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GRAS Notice for 2'-Fucosyllactose for Use in Non-Exempt Infant Formula and Specified Conventional Food Products

PART 1. § 170.225 SIGNED STATEMENTS AND CERTIFICATION

In accordance with 21 CFR §170 Subpart E consisting of §§170.203 through 170.285, Kyowa Hakko Bio Co., Ltd. (Kyowa) hereby informs the United States (U.S.) Food and Drug Administration (FDA) that the intended uses of 2'-fucosyllactose (2'-FL), as manufactured by Kyowa, in non-exempt infant formula, specified conventional food products, and foods for special dietary uses as described in Section 1.3 below, are not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on Kyowa's view that these notified uses of 2'-FL are Generally Recognized as Safe (GRAS). In addition, as a responsible official of Kyowa, the undersigned hereby certifies that all data and information presented in this notice represents a complete and balanced submission that is representative of the generally available literature. Kyowa considered all unfavorable as well as favorable information that is publicly available and/or known to Kyowa and that is pertinent to the evaluation of the safety and GRAS status of 2'-FL as a food ingredient for addition to non-exempt infant formula, specified conventional food products, and foods for special dietary uses as described herein.

Signed,

27 December, 2021
Date

Yoko Kawada, Pharmacist
External Relations Department, Manager
Kyowa Hakko Bio Co., Ltd.
yoko.kawada@kyowa-kirin.co.jp

1.1 Name and Address of Notifier

Kyowa Hakko Bio Co., Ltd.
1-9-2, Otemachi, Choyoda-ku
Tokyo, 100-0004,
Japan

1.2 Common Name of Notified Substance

2'-fucosyllatose (2'-FL)

1.3 Conditions of Use

The proposed food uses and use levels for 2'-FL in the U.S. are presented in Table 1.3-1, whereby food uses are organized according to 21 CFR §170.3 (U.S. FDA, 2020a). As discussed below in Section 3.1, 2'-FL has been concluded to be GRAS for use in term (non-exempt) infant formula, infant and toddler foods (including toddler formula intended for ages 1 to 3 years), conventional foods, and medical foods [GRAS Notices (GRNs) 546, 571, 650, 735, 749, 852, 897, and 932]. The use level of 2'-FL previously concluded to be GRAS in non-exempt term infant formula of 2.4 g/L corresponds to the representative mean concentration of 2'-FL in mature human milk (calculated in GRN 546), and Kyowa intends to use their 2'-FL ingredient in non-exempt term infant formula at this use level of 2.4 g/L. Kyowa's 2'-FL ingredient will be substitutional to other sources of 2'-FL currently on the U.S. market and use levels proposed by Kyowa are the same as those previously concluded to be GRAS, with the exception of cereal and granola bars, whose use level is proposed to be increased from 30 g/kg to 40 g/kg to correspond to the use level concluded to be GRAS for meal replacement bars (40 g/kg) and to align the use levels for products within the category of cereal and granola bars. In addition to the conditions of use previously notified as GRAS for 2'-FL, Kyowa intends to use 2'-FL in the following: bread and baked goods other than gluten-free, protein drinks; chewing gum; non-dairy cream; non-dairy frozen desserts; edible ices, sherbet, and sorbet; energy and protein bars; evaporated and condensed milk; canned fruit; and fruit based desserts (**bolded** in Table 1.3-1). Kyowa also proposes the use of 2'-FL in foods for special dietary uses, specifically, oral nutritional supplements and formula for enteral tube feeding. Oral nutritional supplements are intended for the general population (ages 2 and up). The recommended conditions of use are 2 g 2'-FL/45 g powdered serving or 250 mL ready to consume product, consumed twice per day for a total daily intake of 4 g 2'-FL/day. The use of 2'-FL in enteral tube feeding formula is intended for ages 11 and up and is proposed at a use level of 20 g/L in the final, ready to consume product.

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the United States

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Food Uses[a,b]	Use Levels (g/L or g/kg)
Baked Goods and Baking Mixes	**Breads and baked goods**[c], incl. gluten-free	24 to 48
Beverages and Beverage Bases	Soft drinks (regular and diet)	1.2
	Enhanced, fortified, and flavored waters (incl. carbonated waters)	1.2
	Nondmilk-based meal replacement drinks for weight reduction	5
	Sports, isotonic, and energy drinks	0.8 to 1.2
	Protein drinks	**5**
Breakfast Cereals	Hot breakfast cereals (*e.g.*, oatmeal, grits), instant and RTE	31
	RTE breakfast cereals	
	Puffed cereals	80
	High-fiber cereals	30
	Biscuit-type cereals	20
Chewing Gum	**Chewing gum**	**300**
Coffee and Tea	Coffee	5.0 to 10.0
	Tea	5.0 to 10.0

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the United States

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Food Uses[a,b]	Use Levels (g/L or g/kg)
Dairy Product Analogs	Milk substitutes such as soy milk and imitation milks	1.2
	Beverage whiteners	600
	Non-dairy cream	**80**
	Non-dairy yogurt	12
	Non-dairy frozen desserts	**17**
Frozen Dairy Desserts and Mixes	Frozen desserts incl. ice creams and frozen yogurts, frozen novelties	17
Fruit and Water Ices	**Edible Ices, Sherbet, and Sorbet**	**17**
Gelatins, Puddings, and Fillings	Dairybased puddings, custards, and mousses[d]	17
	Fruit pie filling	14.1
	"Fruit Prep" such as fruit filling in bars, cookies, yogurt, and cakes	30
Grain Products and Pastas	Cereal and granola bars incl. **energy, protein**, and meal replacement bars[e]	40
Infant and Toddler Foods	Term (non-exempt) infant formula (intended for age 0 to <12 months)	2.4 (as consumed)
	Toddler formula (intended for age 1 to 3 years)	2.4 (as consumed)
	Other baby foods for infants and young children	12
	Hot cereals (dry and RTE)	10.9
	Other drinks for young children, incl. yogurt and juice beverages identified as "baby drinks"	1.2 to 10
	Desserts incl. fruit desserts, cobblers, yogurt/fruit combinations ("junior type desserts")	10.9
	Baby crackers, pretzels, cookies, and snack items	57
Jams and Jellies	Jellies and jams, fruit preserves, and fruit butters	60
Milk, Whole, and Skim	Unflavored pasteurized and sterilized milk (whole milk, reduced-fat milk, low-fat milk, non-fat milk; including powdered milks, reconstituted)	1.2
Milk Products	Buttermilk	1.2
	Flavored milk	1.2
	Evaporated and condensed milk	**1.2**
	Milk-based meal replacement beverages for weight reduction	1.2 to 5
	Yogurt	12
	Formula intended for pregnant women ("mum" formulas, -9 to 0 months)[f]	6
Processed Fruits and Fruit Juices	Fruit flavored drinks and ades	1.2
	Fruit juices and nectars	1.2
	Canned fruit	**17**
	Fruit-based desserts	**17**
Processed Vegetables and Vegetable Juices	Vegetable juices and nectars	1.2
Sugar Substitutes	Table-top sweeteners	300
Sweet Sauces, Toppings, and Syrups	Syrups used to flavor milk beverages	7
Foods for Special Dietary Uses	Oral nutritional supplements and enteral tube feeding (11 years and older)[g]	20[h]

Table 1.3-1 Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the United States

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Food Uses[a,b]	Use Levels (g/L or g/kg)

2'-FL = 2'-fucosyllactose; CFR = *Code of Federal Regulations*; FDA= Food and Drug Administration; GRAS= Generally Recognized as Safe; incl.= including; NHANES = National Health and Nutrition Examination Survey; RTE= ready-to-eat; U.S.= United States.

[a] 2'-FL is intended for use in unstandardized products where standards of identity, as established under 21 CFR §130 to 169, do not permit its addition in standardized products.

[b] Additional food uses proposed by Kyowa that have not been previously concluded as GRAS and notified to the U.S. FDA are **bolded**.

[c] The use of 2'-FL was previously concluded to be GRAS in gluten-free breads and baked goods. Kyowa now proposes to also use 2'-FL in conventional breads and baked goods (*i.e.*, both gluten containing and gluten-free products).

[d] Includes gelatin desserts.

[e] The maximum use level of 2'-FL previously concluded to be GRAS in cereal and granola bars (excluding **energy, protein**, and meal replacement bars) was 30 g/kg. The maximum use level of 2'-FL previously concluded to be GRAS in meal replacement bars was 40 g/kg. Kyowa proposes to increase the use level in this category from 30 g/kg to 40 g/kg to correspond to the use level concluded to be GRAS for meal replacement bars (40 g/kg) and to align the use levels for products within the category of cereal and granola bars.

[f] Food codes for "mum formulas" were not available in the 2017-2018 NHANES. This intended use is excluded from the calculation of estimated daily intakes due to absence of consumption data.

[g] Foods for special dietary use were assessed separately from the intended food uses of 2'-FL in conventional foods, as they are intended for supplying a particular dietary need and/or supplementing the intake of a dietary component. Intake of 2'-FL from foods for special dietary use is, therefore, not expected to be cumulative to other dietary sources.

[h] Use level of 20 g/L represents the level of 2'-FL in the final, ready to consume product.

1.4 Basis for GRAS

Pursuant to 21 CFR § 170.30 (a)(b) of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2020b), Kyowa has concluded that the intended uses of 2'-FL as described herein are GRAS on the basis of scientific procedures.

1.5 Availability of Information

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

Yoko Kawada, Pharmacist
External Relations Department
Manager
1-9-2, Otemachi
Chiyoda-ku
Tokyo, 100-0004
Japan

Email: yoko.kawada@kyowa-kirin.co.jp
Phone: +81 70 3145 4956

Should the FDA have any questions or additional information requests regarding this Notification, Kyowa will supply these data and information upon request.

1.6 Freedom of Information Act, 5 U.S.C. 552

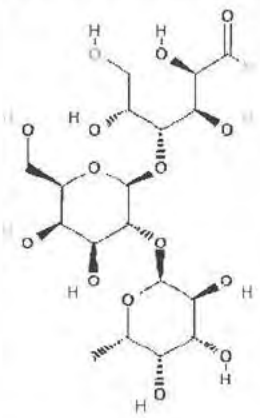
It is Kyowa's view that all data and information presented in Parts 2 through 7 of this Notice do not contain any trade secret, commercial, or financial information that is privileged or confidential, and therefore, all data and information presented herein are not exempted from the Freedom of Information Act, 5 U.S.C. 552.

Part 2. §170.230 Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

2.1 Identity

2'-FL is a fucosylated oligosaccharide composed of 3 monosaccharides, namely L-fucose, D-galactose, and D-glucose. As D-lactose is composed of D-galactose and D-glucose, 2'-FL can also be described as being composed of the disaccharide D-lactose and the monosaccharide L-fucose, linked by an *alpha* (1→2) bond. Kyowa's 2'-FL manufactured by microbial fermentation using a genetically modified strain of *E. coli* W contains by specification ≥82% 2'-FL, with lesser amounts of D-lactose (≤5%), L-fucose (≤1%), D-glucose and D-galactose (≤1%), and difucosyllactose (≤3%). Information regarding the chemical identity of Kyowa's 2'-FL ingredient is provided in Table 2.1-1 below.

Table 2.1-1 Chemical Identity of 2'-FL

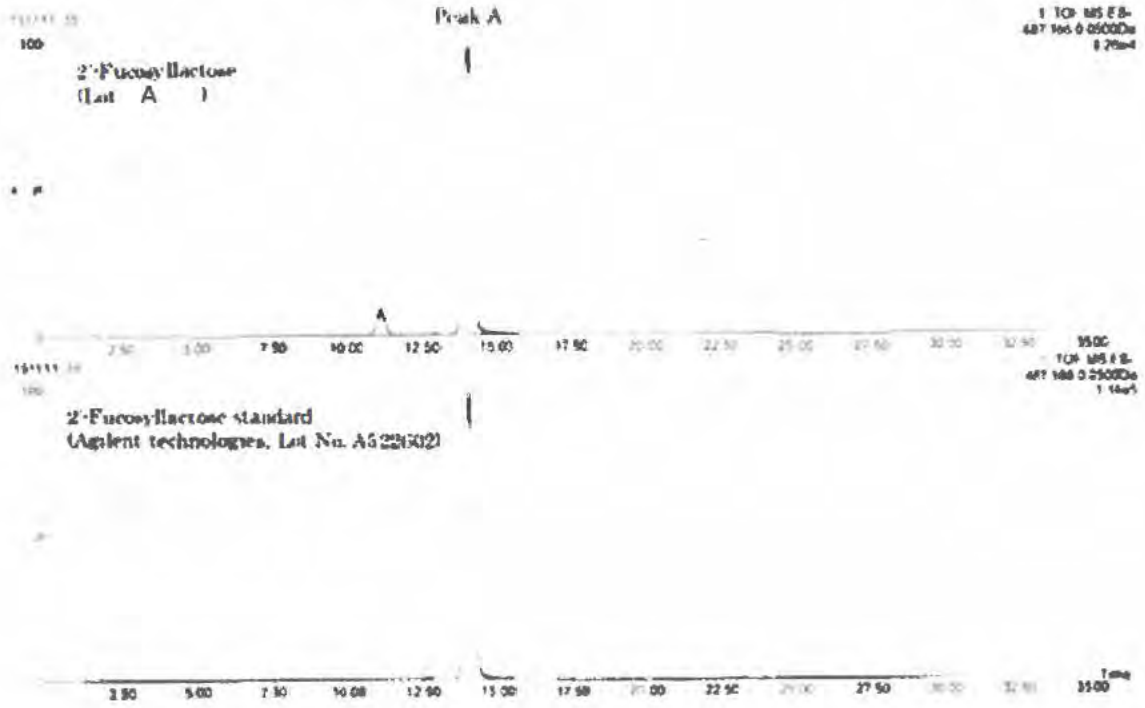
Common Name	2'-Fucosyllactose; 2'-*O*-fucosyllactose
Trade Name	2'-Fucosyllactose; 2'-*O*-fucosyllactose
Common Abbreviations	2' FL; 2 FL; 2FL
International Union of Pure and Applied Chemistry (IUPAC) Name	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Chemical Abstract Service (CAS) Number	41263-94 9
Chemical Formula	$C_{18}H_{32}O_{15}$
Molecular Weight	488.44 g/mol
Structural Formula	

2'-FL was first isolated and identified from human breast milk in the 1950s using chromatographic techniques (Polonowski and Montreuil, 1954; Kuhn *et al.*, 1955; Montreuil, 1956). 2'-FL is the second most abundant free soluble glycan in human milk, following lactose (Castanys-Muñoz *et al.*, 2013). 2'-FL has since been detected in the milk of various mammalian species, although it is most abundant in human milk, and either absent or present at low levels in bovine milk and milk from other domestic commercial animals [summarized in Castanys-Muñoz *et al.* (2013)]. The chemical structure of 2'-FL has been structurally characterized, along with other human milk oligosaccharides (HMOs), by coupling chromatographic separation techniques with more sensitive analytical techniques, such as nuclear magnetic resonance (NMR) spectroscopy (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) and mass spectrometry (Fura and Leary, 1993; Asres and Perreault, 1996; Perreault and Costello, 1999). The structure of 2'-FL has also been elucidated using X-ray crystallography (Kuhn *et al.*, 1956; Svensson *et al.*, 2002).

The chemical and structural identity of Kyowa's 2'-FL produced by fermentation with a genetically modified strain of*E. coli* W (Lot A) was confirmed against 2'-FL isolated from human milk (Agilent technologies, Lot No. A522602) by liquid chromatography-mass spectrometry (LC-MS), proton nuclear magnetic resonance spectroscopy (^{1}H NMR), and carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR). A representative LC-MS chromatogram and a mass spectrum of Kyowa's 2'-FL ingredient demonstrating its purity and identity against 2'-FL obtained from human milk are presented in Figures 2.1-1 and 2.1-2.

Batch analyses of 6 lots of 2'-FL (5 of which were non-consecutive) produced by fermentation with a genetically modified strain of *E. coli* W demonstrate that it is a high purity product (~91 to 96% 2'-FL) with low levels of other structurally related saccharides (see Section 2.3.3).

Figure 2.1-1 **Chromatogram of 2'-FL Produced with a Genetically Modified Strain of *Escherichia coli* W (Lot A) Compared to Standard Isolated from Human Milk (Agilent Technologies, Lot No. A522602)**



2'-FL = 2'-fucosyllactose.

Figure 2.1-2 **Mass Spectrum and Estimated Compositional Formula of 2'-FL (Lot A) Produced with a Genetically Modified Strain of *Escherichia coli* W Compared to Standard Isolated from Human Milk (Agilent Technologies, Lot No. A522602)**

2'-FL = 2'-fucosyllactose.

2.2 Method of Manufacture

2.2.1 Production Microorganism

2.2.1.1 Host

The host organism used in the production of 2'-FL is *E. coli* W. The taxonomic classification of *E. coli* W is listed in Table 2.2.1.1-1.

The *E. coli* W strain is a Gram-negative, rod-shaped, facultative anaerobe that has been used in the industrial production of amino acids for foods, feeds, medicines, and various other applications for nearly 80 years (Archer *et al.*, 2011; UniProt, 2021). *E. coli* W was first isolated from the soil of a cemetery near Rutgers University by Selman A. Waksman, who observed the strain's high sensitivity to streptomycin compared to other isolated *E. coli* strains in his collection, and is thus commonly referred to as "Waksman's strain" or "W strain" (Archer *et al.*, 2011). Early reported uses of *E. coli* W are related to the strain's susceptibility to streptomycin and other antibiotics (Archer *et al.*, 2011).

E.tcoli W is 1 of 4 strains designated safe for laboratory use (K-12, B, C, and W). These 4 strains and their derivatives are designated as Risk Group 1 or Biosafety Level 1 organisms in biological safety guidelines (Archer *et al.*, 2011; ATCC, 2021a), as they are well-characterized and do not cause disease in healthy adult humans (NIH, 2019), and do not colonize the human gut (Bauer *et al.*, 2008). The *E.tcoli* W strain has been deposited in the American Type Culture Collection (ATCC 9637 – ATCC, 2021a), and its genome has been sequenced, annotated, and compared to other safe *E. coli* strains and group B1 commensal/pathogenic *E.tcoli* strains (Archer *et al.*, 2011). Although *E. coli* W has genes that encode pathogenicity determinants, these have been mutationally inactivated or are missing key components required for pathogenicity, similar to other safe strains (Archer *et al.*, 2011). Genomic analyses also confirmed the lack of genes encoding toxins that can be secreted. As such, *E. coli* W is non-pathogenic and non-toxigenic.

Compared to other Risk Group 1 *E.tcoli* strains (K-12, B, and C), *E.tcoli* W has a larger genome [the chromosome is 4,900,968 bp and encodes 4,764 open reading frames (ORFs)], belongs to phylogroup B1 rather than A (both of which are classified as non-pathogenic commensal strains), grows faster, and utilizes a wider range of carbon sources including, unlike the other 3 Risk Group 1 strains, sucrose (Archer *et al.*, 2011; UniProt, 2021). *E.tcoli* W contains 2 plasmids, namely pRK1 and pRK2. The pRK1 plasmid (102,536 bp) encodes 118 genes (114 proteins coding genes, 1 pseudogene, and 3 non-coding RNAs). This plasmid was demonstrated to belong to Incompatibility Group 1 (IncI1) *via* Basic Local Alignment Search Tool (BLAST) analysis and, although genes for antibiotic resistance are typically found on most IncI1 plasmids, the pRK1 plasmid does not encode any antibiotic resistance genes (Archer *et al.*, 2011). The pRK1 plasmid is removed from the host strain and is not present in the production strain. The pRK2 plasmid (5,360 bp; previously sequenced by Štěpánek *et al.*, 2005) encodes 16 genes (15 protein coding genes and 1 non-coding RNA) and is a cryptic ColE1-type plasmid (Archer *et al.*, 2011). The pRK2 plasmid remains in the production strain and no genetic modification was made by Kyowa to the pRK2 plasmid.

Table 2.2.1.1-1 Taxonomic Classification of the Host Organism *Escherichia coli* W

Family	*Enterobacteriaceae*
Genus	*Escherichia*
Species	*Escherichia coli*
Subspecies	Not applicable
Strain	*E. cali* strain W
Culture collection	American Type Culture Collection (ATCC)
Deposition number[a]	ATCC 9637

[a] https://www.atcc.org/products/all/9637.aspx.

2.2.1.2 Host Modifications

The host strain *E.tcoli* W was genetically modified to produce the 2'-FL producing recombinant strain. The production strain was optimized to produce 2'-FL *via* the fermentation of glucose and lactose.

Method of Modification

Target genes are cloned by polymerase chain reaction (PCR) from chromosomal DNA of defined donor organisms and fused to a constitutive promoter originating from *E.tcoli* Wtand expressed at the insertion loci. Desired host modifications are introduced to the *E. coli* W strain in a step-wise manner for the construction of the production strain.

In all instances, genetic modifications were achieved using a modified lambda Red recombination system (Datsenko and Wanner, 2000), a common technique used to make targeted genetic modifications int*E. coli* at loci specified by flanking homology regions including insertions, deletions, and point mutations (Murphy, 1998; Yu *et al*., 2000; Sharan *et al*., 2009). Lambda Red recombination genes are expressed from the Red recombinase pKD46 plasmid under the inducible arabinose promoter (P_{araB}) containing a temperature-sensitive replicon (Datsenko and Wanner, 2000; GenBank Accession No. AY048746t– NCBI, 2021). Following expression of the recombinase enzymes, linear DNA substrates are introduced by electroporation, and recombination is catalyzed by the Lambda-derived proteins (Sharan *et al*., 2009).

Genes of Interest

The production strain contains 1 heterologous gene sequence (encoding α-1,2 fucosyltransferase) originating from *Helicobacter mustelae* (ATCC 43772 – ATCC, 2021b), which is a gastric pathogen of ferrets (Fox *et al*., 1990). The α-1,2 fucosyltransferase gene has been cloned from chromosomal DNA and mutated using site-directed mutagenesis according to Kamada and Koizumi (2007), producing a protein that has α-1,2 fucosyltransferase activity but whose amino acid sequence has at least 1 amino acid that has been deleted, substituted, or added. Although the gene encoding α-1,2 fucosyltransferase is cloned from chromosomal DNA from a pathogenic strain, no unspecified DNA is expected to be associated with the transfer of the gene encoding α-1,2 fucosyltransferase, as the DNA insert is well-characterized and is confirmed to consist ofthe desired sequence only. Furthermore, the expression product α-1,2 fucosyltransferase has a well-defined function in the biosynthesis of 2'-FL and is not associated with any potential toxicity or pathogenic traits of the donor organism.

Host modifications also include the deletion of 5 gene sequences, which serve as insertion loci for the inserted gene described above.

2.2.1.3 Selection of Final Strain

Selection of the final modified *E. coli* W production strain is achieved *via* negative selection using the *Bacillus subtilis sacB* gene coding for levansucrase as a counter-selectable marker (Mizoguchi *et al*., 2007). The enzyme catalyzes the hydrolysis of sucrose and synthesis of high-molecular weight fructose polymers called levans (Pelicic *et al*., 1996). When the *sacB* gene is expressed in *E. coli*, the strain cannot grow in the presence of sucrose.

A marker cassette containing the *sacB* gene (encoding levansucrase, which confers sensitivity to sucrose) and the *cat* gene (which encodes chloramphenicol acetyl transferase and confers chloramphenicol resistance), is inserted into the *E. coli* W strains for the construction of the production strain by homologous recombination following the deletion of the target region using the lamba Red recombinase system. Desired host modifications are then introduced to the *E. coli* W strains in a step-wise manner for the construction of the production strain. Cells expressing the desired genetic traits are selected using the antibiotic resistance marker. The marker cassette is then removed using the lamba Red recombinase system, and cells are plated with sucrose. Cells able to grow in the presence of sucrose are selected as the final strains (as cells containing the *sacB* gene cannot survive in the presence of sucrose). In this manner, the strains containing the desired genetic modifications but lacking the antibiotic resistance gene (which is present in the marker cassette with the *sacB* gene) are selected, as the antibiotic resistance gene cannot be present in order for the cell to survive in the presence of sucrose. After the strains have been selected, PCR at the recombination point is used to verify that all desired genetic modifications have been incorporated.

2.2.1.4 Final Production Strain

The final 2'-FL production strain is non-pathogenic and non-toxigenic and has the same virulence profile as the host organism, as all genetic modifications are well-characterized, confirmed to consist of the desired sequences only, have a well-defined function in the biosynthesis of 2'-FL, and are not associated with any potential toxicity or pathogenic traits of the donor organism. The final 2'-FL production strain is not capable of DNA transfer to other organisms. Therefore, the use of the 2'-FL production strain in the manufacture of Kyowa's 2'-FL is not expected to result in any safety concerns.

2.2.2 Fermentation Media Components, Processing Aids, and Raw Materials

The fermentation media used for culturing the genetically modified strain of *E. coli* W contains nutrient sources and ingredients that are commonly used in microbial growth media. Fermentation media components include ammonia-based salts as a nitrogen source, and vitamins, amino acids, essential mineral mix, trace elements, and yeast extract as sources of nutrients to promote growth.

All additives, processing aids, and food contact materials used in the manufacturing process are food-grade quality or of a higher standard and are used in accordance with an applicable federal regulation, previous conclusion of GRAS status, or have been the subject of an effective food contact notification and are used consistent with current Good Manufacturing Practice (cGMP) requirements. Glucose and lactose are the only carbon sources added to the fermentation medium during the fermentation process. Lactose monohydrate used as a carbon source for the production of 2'-FL by fermentation is derived from cow's milk, which is a major food allergen; however, Kyowa's purification processes (see Section 2.2.3) are effective in the removal of residual proteins and no milk proteins were detected in Kyowa's final 2'-FL ingredient, as described in Section 6.7.

2.2.3 Manufacturing Process

The manufacturing process for Kyowa's 2'-FL is controlled by a Hazard Analysis and Critical Control Points (HACCP) plan and is conducted in accordance with cGMP as established by 21 CFR §117 (U.S. FDA, 2020c). The manufacture of 2'-FL by fermentation with a genetically modified strain of *E.tcoli* W involves 2 main steps: fermentation and purification. Each of the 2 steps is briefly described below, along with a schematic overview of the fermentation and purification processes (see Figure 2.2.3-1).

Fermentation Process

The fermentation process for the production of 2'-FL is conducted using chemically defined nutrient media under sterile conditions.

A master frozen cell bank is prepared for the production strain. Cells from the master cell bank are inoculated to produce the working frozen cell bank. The genetic stability from a minimum of 3 cell passages from the master and working production strain cell bank is verified based on 2'-FL production, cell growth, oxygen consumption, and other functional parameters indicating a change in cell culture behavior.

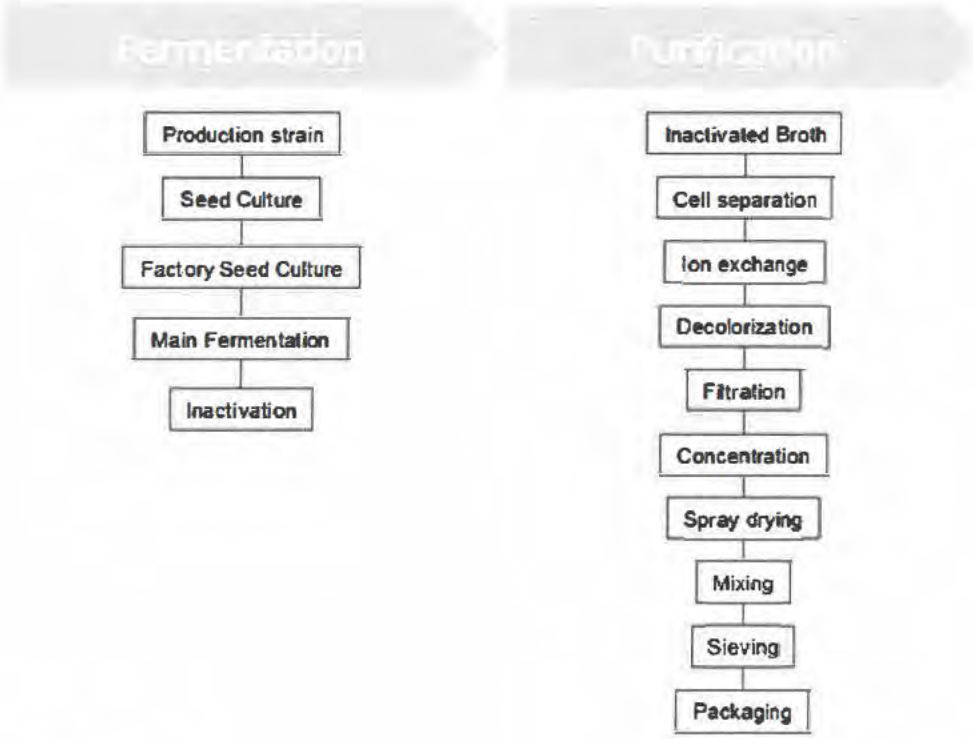
Cells from the working cell bank are then inoculated to produce the production strain flask seed culture. Cells are cultured in the flask seed medium and then transferred to the factory seed medium and cultured. The process conditions are tightly controlled (*e.g.*, time, temperature, pH, and feeding rate). The seed culture step is complete when a specific optical density is reached.

In the main fermentation, the medium is first inoculated with the production strain factory seed culture and fermented in the presence of glucose. Following the depletion of glucose in the culture medium, lactose and glucose are fed to the culture medium. The main fermentation is maintained at a constant temperature until the completion of feeding. During the feeding step, the production strain takes up the lactose and glucose for the synthesis of 2'-FL, which is excreted into the media. As with the initial fermentations, the process conditions of the main fermentation are tightly controlled (*e.g.*, time, temperature, pH, and feeding rate). The production of 2'-FL is stopped *via* heat treatment (sterilization), after which the broth is cooled and acidified.

Purification Process

The intact production strain cells are removed *via* microfiltration. The obtained solution is then passed through a series of cationic resin and anionic resin ion exchangers to remove cations, anions, minerals, and organic impurities. The pH of the effluent is adjusted, and the concentrated solution is decolorized with activated carbon, and then filtered in a series of filtration steps using microfiltration membranes and an ultra-filtration membrane to remove endotoxins, as well as any residual protein, organic impurities, and production organism not removed by the cationic/anionic exchange resins. The obtained solution is then further concentrated, spray-dried, homogenized using an air blender, and then passed through a sieve to remove foreign materials to obtain the final 2'-FL product as outlined in Figure 2.2.3-1.

Figure 2.2.3-1 Schematic Overview of the Fermentation and Purification Processes for 2'-FL Produced Using a Genetically Modified Strain of *Escherichia coli* W



2'-FL = 2'-fucosyllactose.

2.3 Product Specifications and Batch Analysis

2.3.1 Chemical Specifications

Food-grade chemical specifications have been established for 2'-FL and are presented in Table 2.3.1-1. The limits for purity and contaminants established by Kyowa are similar to those established for other 2'-FL ingredients that have been concluded to be GRAS (see Section 6.1 below).

Kyowa's final product is a white to off-white powder with a purity of at least 82% 2'-FL as determined by an in-house validated method [high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD)]. The proposed purity specification for Kyowa's 2'-FL produced using a genetically modified strain of *E. coli* W is similar to the purity of 2'-FL produced using a genetically modified strain of *E. coli* K-12 [*i.e.*, ≥82% dry basis *vs.* ≥82% area under the curve (AUC); as specified in GRN 749] and lower than the purity specifications for other 2'-FL ingredients produced using genetically modified strains of *E. coli* BL21, *E. coli* K12, or from chemical synthesis (*i.e.*, ≥90 to ≥95% purity).

Kyowa also has established limits for potential impurities of the production process, including D-lactose (≤5%), L-fucose (≤1%), difucosyllactose (≤3%), fucosylgalactose (≤3%), and D-glucose and D-galactose (≤1% combined) (all determined by HPLC-PAD). These specifications are similar to or lower than the specification limits for the same compounds in other 2'-FL ingredients (synthetic and microbial source) notified to the U.S. Food and Drug Administration (FDA) (see Table 6.1-1 below). Lactose, fucose, and difucosyllactose are naturally-occurring components of human milk, fucosylgalactose is a naturally-occurring breakdown product of fucosylated oligosaccharides, galactose is a naturally-occurring breakdown product of lactose, and glucose is a naturally-occurring breakdown product of lactose, a common dietary component, and serves as a starting material for the biosynthesis of 2'-FL. The exposures to these carbohydrates from the intended uses of Kyowa's 2'-FL ingredient are expected to be insignificant compared to background exposures and are not expected to pose safety concerns. In addition, residual proteins are specified to be ≤10 mg/kg (determined using a dot-blot method) in Kyowa's final 2'-FL ingredient; this specification is 10 times lower than specifications for residual proteins for other 2'-FL ingredients (synthetic and microbial source) notified to the U.S. FDA.

The specified limit for water content is ≤9 w/w% as determined by Karl-Fischer titration (as specified in the Japanese Pharmacopoeia, 17th Edition, Section 2.48). The ash component of the final product is specified to be ≤0.5 w/w%. The final product is specified to have a pH between 4.0 and 9.0 when analyzed in 5% solution at 20℃.

The specification limits for lead, arsenic, cadmium, and mercury of ≤0.2 mg/kg (individually) and the specification limit for iron of ≤10 mg/kg in the final product are in accordance with the requirements for a food-grade quality ingredient and are similar to the limits for heavy metals for 2'-FL produced using *E. coli* BL21 notified to the U.S. FDA (GRN 571). For synthetic 2'-FL and 2'-FL produced using *E. coli* K-12 (GRNs 546 and 650), specifications for arsenic, cadmium, and mercury are not listed.

Methods of analysis used by Kyowa were obtained from the United States or Japanese Pharmacopeia or were developed in-house. Methods obtained from the United States and Japanese Pharmacopeia are validated for their intended uses. Kyowa uses a validated internal HPLC-PAD method for the identification and quantification of the carbohydrate components. Residual protein is assessed using an internal dot-blot method that has been developed and concluded to be suitable for its intended use by Kyowa.

Table 2.3.1-1 Physical and Chemical Specifications for 2'-FL

Specification Parameter	Specification	Method
Organoleptic		
Appearance	Powder	Visual observation
Color	White to off-white	General Notice, JP[a]
Physicochemical		
Identification	RT of standard ± 3%	HPLC-PAD (internal method)
Purity	≥82% dry basis	HPLC-PAD (internal method)
Water	≤9.0 w/w%	JP 2.48[a]
Ash	≤0.5 w/w%	JP 2.44[a]
Residual protein	≤10 mg/kg (0.001%)	Dot-blot (internal method)
pH (20°C, 5% solution)	4.0 to 9.0	JP 2.54[a]
Other Carbohydrates		
D-lactose	≤5 w/w%	HPLC-PAD (internal method)
L-fucose	≤1 w/w%	HPLC-PAD (internal method)
D-glucose and D-galactose	≤1 w/w%	HPLC-PAD (internal method)
Difucosyllactose	≤3 w/w%	HPLC-PAD (internal method)
Heavy Metals		
Arsenic	≤0.2 mg/kg	USP 233[b]
Cadmium	≤0.2 mg/kg	USP 233[b]
Lead	≤0.2 mg/kg	USP 233[b]
Mercury	≤0.2 mg/kg	USP 233[b]
Iron	≤10 mg/kg	USP 233[b]

2'-FL = 2'-fucosyllactose; HPLC-PAD = high-performance liquid chromatography with pulsed amperometric detection; JP = Japanese Pharmacopoeia; RT = retention time; USP = United States Pharmacopoeia.
[a] Method is consistent with the compendial method specified in 17th edition of the Japanese Pharmacopeia (2016).
[b] Method is consistent with the compendial method specified in the United States Pharmacopeia 35th revision (2011).

2.3.2 Microbiological Specifications

Food-grade microbiological specifications have been established for 2'-FL and are presented in Table 2.3.2-1. The limits for microbiological contaminants established by Kyowa are similar to those established for other 2'-FL ingredients that have been concluded to be GRAS (see Section 6.1 below). Microbial parameters are analyzed using standards from the International Organization for Standardization (ISO). Kyowa also has established a limit of ≤10 endotoxin units (EU)/mg (determined with Section 4.01, kinetic-turbidimetric method, of the Japanese Pharmacopoeia, 17th Edition) for residual endotoxins to ensure that there is no potential contamination from the production organism. Kyowa's specification for residual endotoxins (≤10 EU/mg) is identical to the specification for this parameter for *E. coli* K-12-derived 2'-FL ingredients (GRNs 852 and 735) notified to the U.S. FDA.

Table 2.3.2-1 Microbiological Specifications for 2'-FL

Specification Parameter	Specification	Method
Aerobic plate count	≤1,000 CFU/g	ISO 4833-1:2013
Molds	≤100 CFU/g	ISO 21527-2:2008
Yeasts	≤100 CFU/g	ISO 21527-2:2008
Salmonella[a]	Negative in 100 g	ISO 6579-1:2017
Enterobacteriaceae	Negative in 10 g	ISO 21528-1:2017
Cronobacter spp.[b] *(Enterobacter sakazakii)*	Negative in 100 g	ISO 22964:2017
Listeria monocytogenes	Negative in 25 g	ISO 11290-1:2017
Bacillus cereus	≤50 CFU/g	ISO 7932:2004
Residual endotoxins	≤10 EU/mg	JP 4.01 (kinetic-turbidimetric method)[c]

2'-FL = 2'-fucosyllactose; CFU = colony-forming units; EU= endotoxin units; ISO= International Organization for Standardization; JP= Japanese Pharmacopoeia.

[a] Four individual samples of 25 g are analyzed as per the validated method. All 4 samples must be negative to meet the specification limit.

[b] Ten individual samples of 10 g are analyzed as per the validated method. All 10 samples must be negative to meet the specification limit.

[c] Method is consistent with the compendial method specified in 17th edition of the Japanese Pharmacopeia (2016).

2.3.3 Product Analysis

2.3.3.1 Chemical Analysis of 2'-FL

Analysis of 6 lots of 2'-FL (5 of which were non-consecutive) manufactured by fermentation using a genetically modified strain of *E. coli* W (Lots A, B, C, D, E, and F) demonstrates that the manufacturing process as described in Section 2.2 produces a consistent product that meets specifications. A summary of the chemical batch analyses from 6 lots of 2'-FL produced from a genetically modified strain of *E. coli* W is presented in Table 2.3.3.1-1.

Table 2.3.3.1-1 Summary of Chemical Batch Analyses for the Final 2'-FL Powdered Ingredient Produced with a Genetically Modified Strain of *Escherichia coli* W

Specification Parameter	Specification	Methods of Analysis	Manufacturing Lot					
			A	B	C	D	E	F
Properties								
Appearance	Powder	Visual observation	Complies	Complies	Complies	Complies	Complies	Complies
Color	White to off-white	JP 17; General Notice[a]	Complies	Complies	Complies	Complies	Complies	Complies
pH (20°C, 5% solution)	4.0 to 9.0	JP 2.54[a]	6.3	6.4	6.2	5.7	6.1	6.2
Purity								
Identification	RT of standard ± 3%	HPLC-PAD (internal method)	Complies	Complies	Complies	Complies	Complies	Complies
Purity (% dry basis)	≥82	HPLC-PAD (internal method)	92	92	92	91	96	94
Water (w/w%)	≤9.0	JP 2.48[a]	5.0	3.9	3.9	2.7	2.8	2.3
Ash (w/w%)	≤0.5	JP 2.44[a]	0.2	0.1	0.1	0.0	0.1	0.1
Residual proteins (mg/kg)	≤10	Dot-blot (internal method)[b]	≤1 (LOQ)	≤1 (LOQ)	≤1 (LOQ)	≤1 (LOQ)	≤1 (LOQ)	≤1 (LOQ)
Other Carbohydrates								
D-lactose (w/w%)	≤5	HPLC-PAD (internal method)[c]	3.1	2.7	2.3	2.4	2.1	2.3
L-fucose (w/w%)	≤1	HPLC-PAD (internal method)[c]	≤0.05 (LOQ)	0.1	0.1	0.1	0.1	0.1
D-glucose and D-galactose (w/w%)	≤1	HPLC-PAD (internal method)[c]	0.2	0.1	0.1	0.2	≤0.05 (LOQ)	0.1
Fucosylgalactose (w/w%)	≤3	HPLC-PAD (internal method)[c]	0.8	0.5	0.4	0.9	0.1	0.8
Difucosyllactose (w/w%)	≤3	HPLC-PAD (internal method)[c]	0.5	1.4	0.9	1.0	1.1	1.0
Mass balance	NA	By calculation[d]	96.9	96.9	95.9	95.6	99.6	98.4
Heavy Metals								
Arsenic (mg/kg)	≤0.2	USP 233[e,f]	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)
Cadmium (mg/kg)	≤0.2	USP 233[e,f]	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)
Lead (mg/kg)	≤0.2	USP 233[e,f]	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)
Mercury (mg/kg)	≤0.2	USP 233[e,f]	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)
Iron (mg/kg)	≤10	USP 233[e,f]	0.1	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)	≤0.05 (LOQ)

Table 2.3.3.1-1 Summary of Chemical Batch Analyses for the Final 2'-FL Powdered Ingredient Produced with a Genetically Modified Strain of *Escherichia coli* W

Specification Parameter	Specification	Methods of Analysis	Manufacturing Lot					
			A	B	C	D	E	F

2'-FL = 2'-fucosyllactose; HPLC-PAD = high-performance liquid chromatography with pulsed amperometric detection; JP = Japanese Pharmacopeia; LOQ = limit of quantification; NA = not applicable; RT = retention time; USP = United States Pharmacopeia.

[a] Method is consistent with the compendial method specified in 17th edition of the Japanese Pharmacopeia (2016).

[b] Limit of detection = 1 mg/kg.

[c] LOQ = 0.05 % (w/w).

[d] Sum of 2' FL (purity), ash, D-lactose, L-fucose, D-glucose and D-galactose, fucosylgalactose, and difucosyllactose.

[e] LOQ = 0.05 mg/kg.

[f] Method is consistent with the compendial method specified in the United States Pharmacopeia 35th revision (2011).

2.3.3.2 Microbiological Analysis

Analysis of the same 6 lots of 2'-FL described in Section 2.3.3.1 demonstrates that the product meets the microbiological specifications outlined in Section 2.3.2. A summary of the results of the microbiological analyses for the 6 lots of 2'-FL is presented in Table 2.3.3.2-1.

Table 2.3.3.2-1 Summary of Microbiological Analyses for 2'-FL Produced with a Genetically Modified Strain of *Escherichia coli* W

Specification Parameter	Specification	Methods of Analysis	Manufacturing Lot					
			A	B	C	D	E	F
Aerobic plate count (CFU/g)	≤1,000	ISO 4833-1:2013[a]	<40	<40	<10	110	<10	<10
Molds (CFU/g)	≤100	ISO 21527-2:2008[b]	<100	<100	<100	<100	<100	<100
Yeasts (CFU/g)	≤100	ISO 21527-2:2008[b]	<100	<100	<100	<100	<100	<100
Salmonella[c]	Negative in 100 g	ISO 6579-1:2017[d]	Negative	Negative	Negative	Negative	Negative	Negative
Enterobacteriaceae	Negative in 10 g	ISO 21528-1:2017[e]	Negative	Negative	Negative	Negative	Negative	Negative
Cronobacter spp.[f] *(Enterobacter sakazakii)*	Negative in 100 g	ISO 22964:2017[d]	Negative	Negative	Negative	Negative	Negative	Negative
Listeria monocytogenes	Negative in 25 g	ISO 11290-1:2017[g]	Negative	Negative	Negative	Negative	Negative	Negative
Bacillus cereus (CFU/g)	≤50	ISO 7932:2004[h]	<10	<10	<10	<10	<10	<10
Residual endotoxins (EU/mg)	≤10	JP 4.01 (kinetic-turbidimetric method)	0.009	0.000	0.008	0.003	0.001	0.000

2'-FL = 2'-fucosyllactose; CFU = colony-forming units; EU = endotoxin units; ISO = International Organization for Standardization; JP = Japanese Pharmacopeia.

[a] Limit of detection = 10 CFU/g.

[b] Limit of detection = 100 CFU/g.

[c] Four individual samples of 25 g are analyzed as per the validated method. All 4 samples must be negative to meet the specification limit.

[d] Qualitative test to confirm "absent in 100 g".

[e] Qualitative test to confirm "absent in 10 g".

[f] Ten individual samples of 10 g are analyzed as per the validated method. All 10 samples must be negative to meet the specification limit.

[g] Qualitative test to confirm "absent in 25 g".

[h] Limit of detection = 10 CFU/g.

2.3.3.3 Additional Chemical Characterization

2.3.3.3.1 Absence of Production Organism and DNA

As indicated in Section 2.2.3, the production organism is removed during the purification process of the manufacturing process by a combination of microfiltration, filtration through cationic and anionic exchange resins, and ultra-filtration. The absence of the production organism in the final 2'-FL ingredient is further demonstrated by the lack of detection of *Enterobacteriaceae* in microbiological batch analyses according to internationally recognized methods (ISO 21528-1:2017) (see Table 2.3.3.2-1).

In addition, Kyowa's final 2'-FL ingredient was assessed for residual production organism using a culture method conducted in accordance with the European Food Safety Authority's (EFSA's) *Guidance on the characterization of microorganisms used as feed additives or as production organisms* (EFSA, 2018). Briefly, 3 lots of 2'-FL produced using a genetically modified strain of *E.tcoli* W were cultured in triplicate in Luria-Bertani (LB) medium at 30°C for 44 hours. A PCR analysis was then conducted using primers specific to the production organism. The production organism cultured in LB medium at 30°C overnight was used as a positive control. The results of this test demonstrated that the primers used were appropriate for the detection of the production organism, and that the production organism was absent from the final 2'-FL ingredient.

To confirm the absence of residual production organism-derived DNA in the final product, Kyowa conducted a quantitative PCR analysis using 3 lots of 2'-FL produced using a genetically modified strain of *E. coli* W (assayed in triplicate). The analysis was conducted in accordance with EFSA's *Guidance on the characterization of microorganisms used as feed additives or as production organisms* (EFSA, 2018). The quantitative PCR assay was conducted using primers specific to the production organism, with DNA extracted from the production organism used as a positive control. No residual DNA was detected (limit of quantification of 4 µg/kg or 4 ppb) in the final 2'-FL ingredient.

2.3.3.3.2 Solubility

Kyowa has conducted a solubility test on the final 2'-FL powdered product (Lot D) in accordance with Organisation for Economic Co-operation and Development (OECD) Test Guideline 105 (Flask Method) (*Water solubility*) (OECD, 1995). The results of this study demonstrate that Kyowa's final 2'-FL ingredient has a water solubility of 739 g/L. Given its high solubility in water, no safety concerns related to the particle size of the 2'-FL ingredient are expected.

2.4 Stability of 2'-FL

Kyowa's use of their proprietary strain of *E.tcoli* W as a microbial source for the production of 2'-FL has no effect on the chemical or structural identity of the 2'-FL in the final ingredient. Furthermore, the additional carbohydrates in Kyowa's 2'-FL produced using a genetically modified strain of *E. coli* Wtare comparable to those of the other 2'-FL ingredients on the U.S. market (synthetic or microbial source). Therefore, no changes are expected with respect to the stability profile of Kyowa's 2'-FL ingredient compared to other 2'-FL ingredients currently included on the GRAS inventory list, either during bulk storage or when incorporated into food matrices.

2.4.1 Accelerated Storage Conditions

Although the use of a different production organism is not expected to affect the stability of the final 2'-FL product compared to other 2'-FL ingredients concluded to be GRAS for their intended uses, Kyowa conducted a study to assess the physicochemical and biochemical bulk stability of 3 lots of 2'-FL produced using a genetically modified strain of *E. coli* Wtunder accelerated conditions (temperature of 40 ± 2℃; 75 ± 5% relative humidity) over a 6-month period. 2'-FL was stored in polyethylene bags within an aluminum foil bag, which are similar packaging materials to those intended for storage and distribution of the commercial product. The results are shown in Table 2.4.1-1. 2'-FL was stable throughout the 6-month storage period and remained within specification limits with no significant change in physicochemical parameters (appearance, color, pH, water activity) or biochemical parameters (purity, carbohydrate profile, and water content). As the water activity of 2'-FL was considerably lower than 0.88 at all time-points of evaluation and conditions of storage, microbial growth or toxin formation in Kyowa's 2'-FL ingredient is unlikely (see Section 2.4.3 below for further discussion). The results of this study support a shelf-life of 3 years.

Table 2.4.1-1 Summary of Accelerated Stability Testing (40 ± 2°C; 75 ± 5% Relative Humidity) for 2'-FL Produced with a Genetically Modified Strain of *Escherichia coli* W

Parameter	Specification	Storage Time (months)			
		0	2	4	6
Lot B					
Appearance	Powder	Complies	Complies	Complies	Complies
Color	White to off-white	Complies	Complies	Complies	Complies
Purity (% dry basis)	≥82	92	91	91	91
Water (w/w%)	≤9.0	3.9	3.5	3.5	3.5
Water Activity (Aw)	NA	0.25	-	-	0.18
pH (20℃; 5% solution)	4.0 to 9.0	6.4	6.5	7.0	6.5
D-Lactose (w/w%)[a]	≤5	2.7	2.7	2.7	2.7
L-Fucose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
D-Glucose and D-Galactose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
Fucosyl-Galactose (w/w%)[a]	≤3	0.5	0.5	0.5	0.5
Difucosyllactose (w/w%)[a]	≤3	1.4	1.4	1.4	1.5
Lot C					
Appearance	Powder	Complies	Complies	Complies	Complies
Color	White to off-white	Complies	Complies	Complies	Complies
Purity (% dry basis)	≥82	92	93	93	93
Water (w/w%)	≤9.0	3.9	3.6	3.6	3.6
Water Activity (Aw)	NA	0.25	-	-	0.22
pH (20℃; 5% solution)	4.0 to 9.0	6.2	6.5	6.7	6.6
D-Lactose (w/w%)[a]	≤5	2.3	2.3	2.3	2.4
L-Fucose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
D-Glucose and D-Galactose (w/w%)[a]	≤1	0.1	0.1	0.1	0.1
Fucosyl-Galactose (w/w%)[a]	≤3	0.4	0.4	0.4	0.4

Table 2.4.1-1 Summary of Accelerated Stability Testing (40 ± 2°C; 75 ± 5% Relative Humidity) for 2'-FL Produced with a Genetically Modified Strain of *Escherichia coli* W

Parameter	Specification	Storage Time (months)			
		0	2	4	6
Difucosyllactose (w/w%)[a]	≤3	0.9	0.9	0.9	0.9
Lot E					
Appearance	Powder	Complies	Complies	Complies	Complies
Color	White to off-white	Complies	Complies	Complies	Complies
Purity (% dry basis)	≥82	96	96	99	98
Water (w/w%)	≤9.0	2.8	2.8	2.8	2.8
Water Activity (Aw)	NA	0.14	-	-	TBD
pH (20°C; 5% solution)	4.0 to 9.0	6.1	6.2	5.8	6.2
D-Lactose (w/w%)[a]	≤5	2.1	2.2	2.3	3.6
L-Fucose (w/w%)[a]	≤1	0.1	0.1	0.2	0.4
D-Glucose and D-Galactose (w/w%)[a]	≤1	≤0.05	≤0.05	≤0.05	0.1
Fucosyl-Galactose (w/w%)[a]	≤3	0.1	0.2	0.1	0.3
Difucosyllactose (w/w%)[a]	≤3	1.1	1.1	1.0	0.7

- = batches not analyzed or planned to be analyzed at this time point; 2'-FL = 2'-fucosyllactose; LOQ = limit of quantification; NA = not applicable; TBD = to be determined.
[a] LOQ = 0.05 % (w/w).

2.4.2 Normal Storage Conditions

The recommended storage conditions for 2'-FL are at room temperature. A study to assess the physicochemical and biochemical stability of 1 lot of 2'-FL produced using a genetically modified strain of *E. coli* W (Lot A) under standard room-temperature conditions (25 ± 2°C; 60 ± 5% relative humidity) is ongoing. 2'-FL was stored in polyethylene bags within an aluminum foil bag, which are similar packaging materials used for storage and distribution of the commercial product. The duration of the study is planned to be 36 months (*i.e.*, the proposed shelf-life of 2'-FL), with analyses planned at 0, 2, 4, 6, 9, 12, 18, 24, 30, and 36 months. Results are available to 12 months (see Table 2.4.2-1). The interim results demonstrate that 2'-FL was stable throughout the first 12 months of storage and remained within specification limits, with no significant change in physicochemical (appearance, color, pH, water activity) or biochemical (purity, carbohydrate profile, and water content) parameters. As the water activity of 2'-FL was considerably lower than 0.88 at all time-points of evaluation and conditions of storage, microbial growth, or toxin formation in Kyowa's 2'-FL ingredient is unlikely (see Section 2.4.3 below for further discussion).

Table 2.4.2-1 Summary of Stability Testing of 1 Lot of 2'-FL (Lot A) produced with a Genetically Modified Strain of *Escherichia coli* W Under Standard Conditions (25 ± 2°C; 60 ± 5% Relative Humidity)

Parameter	Specification	Storage Time (months)					
		0	2	4	6	9	12
Appearance	Powder	Complies	Complies	Complies	Complies	Complies	Complies
Color	White to off-white	Complies	Complies	Complies	Complies	Complies	Complies
Purity (% dry basis)	≥82	92	92	92	91	96	94
Water (w/w%)	≤9.0	5.0	3.9	3.8	3.7	3.7	3.5
Water Activity (Aw)	NA	0.22	-	-	0.26	-	0.19
pH (20°C; 5% solution)	4.0 to 9.0	6.3	6.6	7.8	7.8	8.2	7.8
D-Lactose (w/w%)[a]	≤5	3.1	2.6	2.8	2.8	3.0	2.7
L-Fucose (w/w%)[a]	≤1	≤0.05	≤0.05	≤0.05	≤0.05	≤0.05	≤0.05
D-Glucose and D-Galactose (w/w%)[a]	≤1	0.2	0.2	0.2	0.2	0.2	0.2
Fucosyl-Galactose (w/w%)[a]	≤3	0.8	0.7	0.7	0.7	0.8	0.7
Difucosyllactose (w/w%)[a]	≤3	0.5	0.4	0.5	0.5	0.5	0.5

- = not measured; 2'-FL = 2'-fucosyllactose; LOQ = limit of quantification; NA = not applicable.
[a] LOQ = 0.05 % (w/w).

2.4.3 Microbiological Stability

It has been noted that microbial survival and growth in composite products and foods in general is affected by factors including low water activity, whereby, in general, foods with measured water activity of <0.88 prevent the growth and formation of toxins by food-borne pathogenic bacteria (EFSA, 2012). Kyowa therefore measured the water activity of 2'-FL after 0 and 6 months of storage under accelerated conditions (40 ± 2°C; 75 ± 5% relative humidity), and after 0, 6, and 12 months of storage under standard conditions (25 ± 2°C; 60 ± 5% relative humidity). Additional analyses are planned at 18, 24, 30, and 36 months of storage under standard conditions. As shown above in Tables 2.4.1-1 and 2.4.2-1, the water activity of 2'-FL was considerably lower than 0.88 at all time-points of evaluation and conditions of storage, with values not exceeding 0.26. The low water content of the analyzed batches of 2'-FL indicate also that the storage packaging prevents water absorption by the 2'-FL ingredient. Based on the low water content and water activity values, microbial growth or toxin formation in Kyowa's 2'-FL ingredient is unlikely.

Part 3. §170.235 Dietary Exposure

3.1 Current Regulatory Status

2'-FL is not currently regulated under Title 21 of the CFR for use in food or beverages. A number of 2'-FL ingredients have been concluded to be GRAS for use as ingredients in infant formula and various conventional food products. To date, 13 GRAS Notices for 2'-FL have been notified to the offices of the U.S. FDA under the voluntary notification procedure, 2 of which are currently pending, 2 of which the FDA has ceased to evaluate, and 9 of which the U.S. FDA responded with no questions (see Table 3.1-1 below). A GRAS Notice for 2'-FL and difucosyllactose (2'-FL/DFL) also was identified in the FDA's GRAS Notice Inventory and was filed without objection by the agency under GRN 815. In the GRAS Notices to which the U.S. FDA responded with no questions, 2'-FL is generally intended for use as an ingredient in term (non-exempt) infant formula and toddler formula at levels of up to 2.4 g/L, in exempt hypoallergenic infant formula and hypoallergenic toddler formula (*i.e.,* hydrolyzed cow milk protein- and amino acid-based) at a level of 2 g/L formula, in baby foods and beverages for young children at levels ranging between 1.2 and 57 g/kg or L, in conventional foods at levels ranging between 0.8 and 600 g/kg or L, and medical foods at a level of 20 g/kg (GRN 735 only).

The same synthetic and microbial sources of 2'-FL have gained novel food approval in the European Union (EU) according to specifications and conditions of use listed in Commission Implementing regulation (EU) 2017/2470 (EU, 2017). In the EU, 2'-FL is generally permitted for use in the same food categories as the U.S., but at slightly lower use levels. For example, 2'-FL is permitted in infant formula and follow-on formula (maximum level of 1.2 g/L), baby foods and beverages for young children (levels ranging between 1.2 and 12 g/kg or L), conventional foods (level ranging between 1.2 and 400 g/kg or L), and foods for special medical purposes (according to particular nutritional requirements). 2'-FL is also permitted in food supplements not intended for infants in the EU (1.2 g/day for young children and 3.0 g/day for the general population).

Table 3.1-1 GRAS Notices for 2'-FL Filed with No Objections

GRN Number	Applicant	Ingredient	Source	Purity	Intended Food Uses and Use Levels (g/kg or g/L)
546	Glycom A/S	2'-FL	Synthetic	≥95% on a dry matter basis	Intended for use as an ingredient in term formula at a maximum level of 2.4 g/L. Also intended for use in baby foods at levels ranging between 1.2 and 12 g/L, and in conventional foods [including baked goods and baking mixes, beverages and beverages bases, coffee and tea, dairy product analogs, infant and toddler foods, grain products and pastas, milk (whole and skim), processed fruits and juices, processed vegetables and juices, and sugar substitutes] at levels ranging between 1.2 and 600 g/kg.
571	Jennewein Biotechnologie, GmbH	2'-FL	Fermentation [*Escherichia coli* BL21 (DE3) #1540]	≥90%	Intended for use as an ingredient in infant and toddler formula at a maximum level of 2 g/L.

Table 3.1-1 GRAS Notices for 2'-FL Filed with No Objections

GRN Number	Applicant	Ingredient	Source	Purity	Intended Food Uses and Use Levels (g/kg or g/L)
650	Glycom A/S	2'-FL	Fermentation (modified *Escherichia coli* K-12 DH1; strain SCR6)	≥94%	Intended for use as an ingredient in term infant formula at a maximum level of 2.4 g/L. Also intended for use in baby foods at levels ranging between 1.2 and 12 g/L, and in conventional foods [including beverages and beverages bases, dairy product analogs, grain products and pastas, milk (whole and skim), milk products, processed fruits and juices, and processed vegetables and juices] at levels ranging between 1.2 and 40 g/kg.
735	Glycosyn, LLC and Friesland Campina Domo B.V.	2'-FL	Fermentation (*Escherichia coli* K-12 strain E638)	≥90%	Intended for use as an ingredient in non-exempt infant formula and follow-on formula at a maximum level of 2.4 g/L. Also intended for use in baby foods at levels ranging between 10.9 and 57 g/kg, in conventional foods [including beverages and beverage bases, breakfast cereals, dairy product analogs, frozen dairy desserts and mixes, gelatins, puddings, and fillings, grain products and pastas, jams and jellies, milk, whole and skim, milk products, processed fruits and fruit juices, sweet sauces, toppings, and syrups] at levels ranging between 0.8 and 80 g/kg, and in medical foods at a maximum level of 20 g/kg.
749	DuPont Nutrition & Health	2'-FL	Fermentation (*Escherichia coli* K-12 MG1655 INB3051)	≥82%	Intended for use as an ingredient in term infant formula and toddler formula at a maximum level of 2.4 g/L. Also intended for use in baby foods at levels ranging between 1.2 and 12 g/kg.
815	Glycom A/S	2'-FL/DFL	Fermentation (*Escherichia coli* K-12 DH1)	2'-FL ≥75% DFL ≤20%	Intended for use as an ingredient in term infant formula and toddler formula at a maximum level of 1.6 and 1.2 g/L, respectively. Also intended for use in baby foods at levels ranging between 1.2 and 10 g/kg and in conventional foods [beverage and beverage bases; grain products and pastas; milk (whole and skim), and milk products] at levels ranging from 2.0 to 40 g/kg.
852	BASF SE	2'-FL	Fermentation (*Escherichia coli* K-12 strain LU20297)	≥90%	Intended for use as an ingredient in non-exempt infant and follow-on formula at a maximum level of 2.4 g/L. Also intended for use in baby foods at levels ranging between 2.0 and 57 g/kg and in conventional foods [beverages and beverage bases; breakfast cereals; dairy product analogues; frozen dairy desserts and mixes; gelatins, puddings, and fillings; grain products and pastas; jams and jellies; milk, whole and skim; milk products; processed fruits and fruit juices; sweet sauces, toppings, and syrups] at levels ranging between 0.8 and 80 g/kg.

Table 3.1-1 GRAS Notices for 2'-FL Filed with No Objections

GRN Number	Applicant	Ingredient	Source	Purity	Intended Food Uses and Use Levels (g/kg or g/L)
897	DuPont Nutrition and Health	2'-FL	Fermentation (*Escherichia coli* K-12 strain MG1655 INB000846)	>96%	Intended for use as an ingredient in term infant formula, toddler formulas, baby foods and beverages for young children at levels ranging from 0.24 to 2.04 g/serving, and baked goods and baking mixes; non-alcoholic beverages and beverage bases; breakfast cereals; milk products; dairy product analogs; processed fruits and fruit juices; processed vegetables and vegetable juices; and tube-feeding formulas at levels ranging from 1.2 to 40 g/kg.
929	Jennewein Biotechnologie GmbH	2'-FL	Fermentation (*Escherichia coli* BL21 strain DE3)	≥90%	Intended for use as an ingredient in exempt hypoallergenic infant formula for term infants and hypoallergenic toddler formula at a level of 2 g/L of formula, as consumed. The notifier states that hypoallergenic formula includes both extensively hydrolyzed cow milk protein- and amino acid-based formula.
932	Advanced Protein Technologies Corp.	2'-FL	Fermentation (*Corynebacterium glutamicum* APC199)	≥94%	Intended for use as an ingredient at a level of 2.4 g/L of formula as consumed in milk- and soy-based, non-exempt infant formula for term infants; at a level of 2.4 g/L in drinks for toddlers and children ages 1-3 years, as consumed; at levels ranging from 0.24-1.2 g/serving in infant and toddler foods; and at levels ranging from 0.28-1.2 g/serving in beverage and beverages bases, breakfast cereals, dairy product analogs, frozen dairy desserts and mixes, gelatins, puddings, fillings, grain products and pastas, jams and jellies, milk and milk products, processed fruits and fruit juices, and sweet sauces, toppings and syrup.

2'-FL = 2'-fucosyllactose; DFL = difucosyllactose; GRAS= Generally Recognized as Safe; GRN = GRAS Notice.

3.2 History of Safe Consumption

The history of safe consumption of 2'-FL has been extensively discussed in previous GRAS Notices (particularly in GRNs 546 and 650). In summary, 2'-FL is a naturally occurring oligosaccharide in human milk, synthesized from lactose in the mammary gland (*via* fucosylation by the enzyme α(1,2)fucosyltransferase (FUT2) (Castanys-Muñoz *et al.*, 2013). 2'-FL has been detected in maternal serum during pregnancy, its levels varying according to gestational age and Secretor status (Jantscher-Krenn *et al.*, 2019). It has also been detected in cord serum samples and demonstrated to cross the human placenta *ex vivo* (Hirschmugl *et al.*, 2019). It is the second most abundant glycan in human milk following lactose, and the most abundant human milk oligosaccharide (Castanys-Muñoz *et al.*, 2013). Several factors influence the levels of 2'-FL in human milk, including the stage of lactation (levels generally decrease as lactation progresses), ethnicity, geographical location, and genetic traits (Lewis blood groups and Secretor status) (Erney *et al.*, 2000; Gabrielli *et al.*, 2011; Galeotti *et al.*, 2012; McGuire *et al.*, 2017).

Maternal Secretor and Lewis status are the main sources of variation in HMO milk levels. The Secretor gene (*FUT2*) encodes α(1,2)-fucosyltransferase, the enzyme that catalyzes the synthesis of 2'-FL, whereas the Lewis gene (*FUT3*) encodes α(1,3)-fucosyltransferase and α(1,4)-fucosyltransferase, enzymes that catalyze α1-3 or α1-4 linkages instead (Kumazaki and Yoshida, 1984; Thurl *et al*., 1997, 2010; Stahl *et al*., 2001). Both genes have a functional dominant allele and a non-functional recessive allele, resulting in 4 genetic groups:

1. Secretors, Lewis-positive (Se+/Le+);
2. Non-Secretors, Lewis-positive (Se-/Le+);
3. Secretors, Lewis-negative (Se+/Le-); and
4. Non-Secretors, Lewis-negative (Se-/Le-).

As expected, 2'-FL levels in maternal milk are higher in Secretors *versus* non-Secretors (Groups 1 and 3). Furthermore, 2'-FL levels are higher in Secretors who are Lewis-negative *versus* Secretors who are Lewis-positive (reviewed by Castanys-Muñoz *et al*., 2013).

A comprehensive literature review of publications reporting on 2'-FL maternal milk levels has previously been conducted by Glycom (GRN 546 Glycom A/S, 2014; U.S. FDA, 2015a). The average concentrations of 2'-FL in pooled mature human milk collected from Lactation Days 5 to 100 (considering different demographic groups and phenotypes) were determined to range between 1.10 and 4.26 g/L, whereby 2.4 g/L was determined to be representative of the mean concentration of this oligosaccharide in human milk (Chaturvedi *et al.*, 1997, 2001a; Thurl *et al*., 1997, 2010; Coppa *et al.*, 1999, 2011; Nakhla *et al.*, 1999; Erney *et al*., 2000, 2001; Sumiyoshi *et al.*, 2003; Morrow *et al*., 2004; Leo *et al.*, 2010; Asakuma *et al*., 2011; Galeotti *et al.*, 2012, 2014; Bao *et al.*, 2013; Smilowitz *et al.*, 2013; Hong *et al.*, 2014). This mean level corresponds to concentrations of 2'-FL that have been previously concluded to be GRAS for use in infant formula. Considering individual milk types, the concentration of 2'-FL was reported to be highest in milk from Secretor mothers with a Lewis-negative phenotype collected within 1 month of lactation, ranging from 5.83 to 8.52 g/L at the mean (Gabrielli *et al*., 2011; Galeotti *et al.*, 2012). A concentration of 7.0 g/L was determined to be representative of maximum 2'-FL levels in mature breast milk of mothers with the Secretor phenotype (GRN 546 Glycom A/S, 2014; U.S. FDA, 2015a). In pooled colostrum collected from Lactation Days 0 to 5, the average concentration of 2'-FL was determined to be 3.0 g/L, with levels typically ranging from 1.1 to 4.9 g/L and concentrations as high as 8.4 g/L reported in milk from Secretor mothers. Milk levels reported to occur in human milk (as determined in GRN 546) are summarized in Table 3.2-1.

Exposure to 2'-FL on a body weight basis was calculated based on maternal milk levels described above, assuming a standard infant body weight of 6.7 kg (WHO Growth Chart[1]; average of 50th percentile for boys and girls at 4 months) and a milk consumption of 1.2 L/day (Butte *et al.*, 2002; da Costa *et al.*, 2010; Nielsen *et al*., 2011; EFSA, 2013). The resulting intake of 2'-FL from mature human milk by infants was determined to range between 197 and 770 mg/kg body weight/day, with a maximum intake of up to 1,254 mg/kg body weight/day among infants from Secretor mothers. In newborns, intake was derived in GRN 546 assuming a body weight of 3.4 kg (WHO, 2015), and a milk consumption of 250 mL/day during the first 5 days postpartum (Hester *et al.*, 2012). The average intake of 2'-FL from colostrum was estimated to range between 80 and 360 mg/kg body weight/day, with a maximum intake of up to 620 mg/kg body weight/day in newborns from Secretor mothers (GRN 546 Glycom A/S, 2014; U.S. FDA, 2015a). Estimated daily intakes of 2'-FL from human milk are presented on an absolute and body weight basis in Table 3.2-1.

[1] https://www.cdc.gov/growthcharts/who_charts.htm.

Table 3.2-1 2'-FL Levels in Human Milk and Corresponding Estimated Daily Intake

Lactation Stage	2'-FL Levels in Milk (g/L)			Estimated Daily Intake of 2'-FL (g/person/day)			Estimated Daily Intake of 2'-FL (mg/kg bw/day)		
	Mean (All)	Mean Range (All)	Max (Secretors)	Mean (All)	Mean Range (All)	Max (Secretors)	Mean (All)	Mean Range (All)	Max (Secretors)
Colostrum (LD 0 to 5)	3.0	1.1 to 4.9	8.4	0.75[a]	0.28 to 1.2[a]	2.1[a]	221[b]	80 to 360[b]	620[b]
Mature Milk (mean: LD 5 to 100; max: LD ≤30)	2.4	1.1 to 4.3	7.0	2.9[c]	1.3 to 5.2[c]	8.4[c]	430[d]	197 to 770[d]	1,254[d]

2'-FL = 2'-fucosyllactose; bw = body weight; LD = Lactation Day; Max = maximum.
[a] Assuming a milk consumption of 0.25 L/day.
[b] Assuming a bw of 3.4 kg and milk consumption of 0.25 L/day.
[c] Assuming a milk consumption of 1.2 L/day.
[d] Assuming a bw of 6.7 kg and milk consumption of 1.2 L/day.

In a newly identified study concentrations of 2'-FL of up to 3.7 and 5.6 g/L were reported in Chinese and Malaysian mothers, respectively (Ma *et al.*, 2018). As previously reported, 2'-FL levels in milk decreased as lactation progressed and Secretor status influenced 2'-FL concentrations, with non-detectable 2'-FL levels in breast milk samples from non-Secretors.

In addition to human milk, it has been previously reported that 2'-FL occurs in the maternal circulation and urine as early as at the end of the first trimester (Hallgren *et al.*, 1977). 2'-FL was recently detected in the amniotic fluid at birth, indicating that the fetus is exposed to 2'-FL *in utero* (Wise *et al.*, 2018).

As indicated in Section 2.1, 2'-FL is either absent or detected at low levels in bovine milk and milk from other domestic commercial animals (reviewed by Castanys-Muñoz *et al.*, 2013). Thus, exposure from the consumption of bovine and other milks is expected to be negligible. For example, in comparison to human milk, bovine milk contains lower oligosaccharide levels and less complex oligosaccharides (Tao *et al.*, 2009; Aldredge *et al.*, 2013; Urashima *et al.*, 2013), little of which are fucosylated (Gopal and Gill, 2000; Aldredge *et al.*, 2013).

3.3 Nutritional Purpose for Use in Non-Exempt Infant Formula

2'-FL is intended to be added as a nutritional ingredient to non-exempt infant formula, as well as specified foods and beverages as described in Section 1.3 and defined under 21 CFR §170.3(n) (U.S. FDA, 2020a).

As indicated in Section 3.2, 2'-FL is a naturally-occurring oligosaccharide in human milk and is the most abundant human milk oligosaccharide (Castanys-Muñoz *et al.*, 2013). Human milk offers all essential nutrients for infant growth and development. For this reason, infant formulae are formulated to match the nutrient composition of human milk as closely as possible. Kyowa notes that human milk is a complex fluid containing over 150 HMOs, and is proposing the addition of 2'-FL to non-exempt term infant formula to provide a source of 2'-FL for formula-fed infants.

3.4 Estimated Dietary Consumption of 2'-FL Based Upon Intended Food Uses

3.4.1 Methodology

An assessment of the anticipated intake of 2'-FL as an ingredient under the intended conditions of use (see Table 1.3-1) was conducted using data available in the 2017-2018 cycle of the U.S. National Center for Health Statistics' National Health and Nutrition Examination Survey (NHANES) (CDC, 2021a,b; USDA, 2021). The assessment included all uses previously concluded to be GRAS for 2'-FL in order to provide cumulative estimates of intake.

The NHANES data are collected and released in 2-year cycles with the most recent cycle containing data collected in 2017-2018. Information on food consumption was collected from individuals *via* 24-hour dietary recalls administered on 2 non-consecutive days (Day 1 and Day 2). Sample weights were incorporated with NHANES data to compensate for the potential under-representation of intakes from specific populations and allow the data to be considered nationally representative. The NHANES data were employed to assess the mean and 90th percentile intake of 2'-FL for each of the following population groups:

- Infants, ages 0 to 6 months;
- Infants, ages 7 to <12 months;
- Toddlers, ages 1 to 3 years;
- Children, ages 4 to 11 years;
- Female teenagers, ages 12 to 19 years;
- Male teenagers, ages 12 to 19 years;
- Female adults of childbearing age, ages 14 to 50 years;
- Female adults, ages 20 to 64 years;
- Male adults, ages 20 to 64 years;
- Elderly, agest≥ 65 years; and
- Total population (≥2 years, gender groups combined[2]).

Consumption data from individual dietary records, detailing food items ingested by each survey participant, were collated by computer and used to generate estimates for the intake of 2'-FL by the U.S. population[3]. Estimates for the daily intake of 2'-FL represent projected 2-day averages for each individual from Day 1 and Day 2 of NHANES 2017-2018 (*i.e.*, a value was established for each person). From these average amounts, a distribution was established from which the mean and percentile intake estimates for the cohort of interest were determined, which incorporated survey weights in order to provide representative intakes for the entire U.S. population. "*Per capita*" intake refers to the estimated intake of 2'-FL averaged over all individuals surveyed, regardless of whether they consumed food products in which 2'-FL is proposed for use, and therefore includes individuals with "zero" intakes (*i.e.*, including individuals who reported no intake of food products containing 2'-FL during the 2 survey days). "Consumer-only" intake refers to the estimated intake of 2'-FL by only those individuals who reported consuming food products in which 2'-FL is proposed for use on either Day 1 or Day 2 of the survey.

[2] Although there are 2 female adult population groups, female adults were not double counted within the total population intake results.

[3] Statistical analysis and data management were conducted in DaDiet Software (Dazult Ltd., 2018). DaDiet Software is a web-based software tool that allows accurate estimate of exposure to nutrients and to substances added to foods, including contaminants, food additives and novel ingredients. The main input components are concentration (use level) data and food consumption data. Data sets are combined in the software to provide accurate and efficient exposure assessments.

The estimates for the intake of 2'-FL were generated using the maximum use level indicated for each intended food use, as presented in Table 1.3-1, together with food consumption data available from the 2017-2018 NHANES datasets. The results for these assessments are presented in Section 3.4.2.

3.4.2 Results of Intake Estimates for 2'-FL

3.4.2.1 Estimated Daily Intake of 2'-FL from All Proposed Conditions of Use

A summary of the estimated daily intake of 2'-FL from all proposed food uses is provided in Table 3.4.2.1-1 on an absolute basis (g/person/day), and in Table 3.4.2.1-2 on a body weight basis (mg/kg body weight/day).

The percentage of consumers was high among all age groups evaluated in the current intake assessment; greater than 72.1% of the population groups consisted of consumers of food products in which 2'-FL is currently proposed for use (see Table 3.4.2.1-1). With the exception of infants 0 to 6 months of age, the proportion of consumers was close to or equal to 100.0% in all population groups. The consumer-only estimates are more relevant to risk assessments as they represent exposures in the target population; consequently, only the consumer-only intake results are discussed in detail herein.

Among the total population (2 years and older), the mean and 90th percentile consumer-only intakes of 2'-FL were determined to be 9.4 and 17.4 g/person/day, respectively. Of the individual population groups, the elderly were determined to have the greatest mean and 90th percentile consumer-only intakes of 2'-FL on an absolute basis, at 11.7 and 20.6 g/person/day, respectively, while infants 0 to 6 months had the lowest mean and 90th percentile consumer-only intakes of 2.4 and 4.3 g/person/day, respectively (see Table 3.4.2.1-1).

Table 3.4.2.1-1 Summary of the Estimated Daily Intake of 2'-FL from All Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (g/day)		Consumer-Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants[a]	0 to 6 m	1.7	3.8	75.3	133	2.4	4.3
Infants[a]	7 to <12 m	4.7	8.0	100	124	4.7	8.0
Toddlers	1 to 3 y	5.0	8.6	99.9	414	5.0	8.6
Children	4 to 11 y	6.4	10.8	99.9	889	6.4	10.8
Female Teenagers	12 to 19 y	6.8	12.6	99.3	446	6.8	12.6
Male Teenagers	12 to 19 y	7.4	14.8	99.7	440	7.4	14.8
Females of childbearing age	14 to 50 y	10.1	17.1	99.7	1,354	10.2	17.1
Female Adults	20 to 64 y	10.5	20.4	99.7	1,626	10.5	20.4
Male Adults	20 to 64 y	9.3	19.8	99.3	1,424	9.3	20.0
Elderly	≥65 y	11.7	20.6	99.6	1,057	11.7	20.6
Total Population	≥2 y	9.4	17.4	99.6	6,143	9.4	17.4

2'dFL = 2' fucosyllactose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S.d= United States; y = years.

[a] Consumption in infants also includes intakes from hypoallergenic infant formula, which is not currently being considered as an intended use.

On a body weight basis, the total population (2 years and older) mean and 90th percentile consumer-only intakes of 2'-FL were determined to be 143 and 298 mg/kg body weight/day, respectively. Among the individual population groups, infants 7 to <12 months were identified as having the highest mean and 90th percentile consumer-only intakes of any population group, of 520 and 886 mg/kg body weight/day, respectively. Male adults had the lowest mean and 90th percentile consumer-only intakes of 105 and 209 mg/kg body weight/day, respectively (see Table 3.4.2.1-2).

Table 3.4.2.1-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL from All Proposed Food Uses in the U.S. by Population Group (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)		Consumer-Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants[a]	0 to 6 m	256	555	72.1	133	355	570
Infants[a]	7 to <12 m	520	886	100	124	520	886
Toddlers	1 to 3 y	367	640	99.9	404	368	640
Children	4 to 11 y	225	411	99.9	887	225	411
Female Teenagers	12 to 19 y	113	221	99.3	439	114	221
Male Teenagers	12 to 19 y	116	235	99.7	437	116	235
Females of childbearing age	14 to 50 y	139	263	99.7	1,342	140	263
Female Adults	20 to 64 y	142	273	99.7	1,619	142	273
Male Adults	20 to 64 y	104	209	99.3	1,416	105	209
Elderly	≥65 y	146	261	99.6	1,038	146	261
Total Population	≥2 y	143	298	99.6	6,089	143	298

2'-FL = 2'-fucosyllactose; m = months; n = sample size; NHANES = National Health and Nutrition Examination Survey; U.S.= United States; y = years.

[a] Consumption in infants also includes intakes from hypoallergenic infant formula, which is not currently being considered as an intended use.

The total U.S. population (except infants 0 to <12 months of age) was identified as being significant consumers of "Breads and baked goods, including gluten-free" (80 to 93% consumers), "Unflavored milk" (42 to 80% consumers), "Fruit juices and nectars" (23 to 59% consumers), "Ready-to-eat breakfast cereals" (23 to 59% consumers), "Soft drinks (regular and diet)" (21 to 54% consumers), and "Fruit flavored drinks" (17 to 45% consumers). Infants 0 to 6 months of age were identified as being significant consumers of "Term infant formula" (58% consumers), whereas infants 7 to <12 months were identified as being significant consumers of "Other baby foods for infants and young children" (64% consumers) and "Term infant formula" (60% consumers).

In terms of contribution to total mean intake of 2'-FL, "Breads and baked goods, including gluten-free" (which contributed 28 to 57% to total mean intakes) and "Beverage whiteners" (which contributed 1 to 47% to total mean intakes) were the main source of intake across the total U.S. population (except in infants 0 to <12 months of age); all other food uses contributed less than 16% to total mean 2'-FL intakes. In infants 0 to 6 months of age, "Term infant formula" was the main source of intake (contributed 65% to total mean intakes), whereas in infants 7 to <12 months of age "Other baby foods for infants and young children" (contributed 25% to total mean intakes) and "Term infant formula" (contributed 20% to total mean intakes) were the main sources of intake.

3.4.2.2 Estimated Daily Intake of 2'-FL from Infant and Toddler Formulas

A summary of the estimated daily intake of 2'-FL in younger population groups from the maximum proposed use levels in non-exempt infant formula and toddler formula, as well as from use in hypoallergenic infant formula (which is not a proposed food use) is provided in Table 3.4.2.2-1 on an absolute basis (g/person/day), and in Table 3.4.2.2-2 on a body weight basis (mg/kg body weight/day).

The proportion of consumers ranged between 64.7 to 69.6% in infants, whereas only 4.1 to 4.2% of toddlers were determined to be consumers of the formulas (see Table 3.4.2.2-1). It should be noted that intake estimates derived for toddlers may not be statistically reliable, as only 18 toddlers from the NHANES 2017-2018 cycle were identified as consuming non-exempt infant formula, toddler formula, or hypoallergenic infant formula. As a result, estimates for this population group are presented in Tables 3.4.2.2-1 and 3.4.2.2-2, but not further discussed.

The mean and 90th percentile consumer-only intakes of 2'-FL from use in non-exempt infant formula, toddler formula, and hypoallergenic infant formula were highest in infants 0 to 6 months of age, at 1.87 and 2.96 g/person/day, respectively (see Table 3.4.2.2-1). Intake estimates were also highest in this population group on a body weight basis, at up to 295 and 494 mg/kg body weight/day at the mean and 90th percentile, respectively (see Table 3.4.2.2-2).

Table 3.4.2.2-1 Summary of the Estimated Daily Intake of 2'-FL from Infant Formula and Toddler Formula in the U.S. in Infant and Toddler Populations (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (g/day)[a]		Consumer-Only Intake (g/day)[a]			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	1.21	2.70	64.7	118	1.87	2.96
Infants	7 to <12 m	1.11	2.48	69.6	84	1.60	2.68
Toddlers	1 to 3 y	0.02*	NA	4.1	18	0.58*	1.17*

2'-FL = 2'-fucosyllactose; m = months; n = sample size; NA= not applicable; NHANES = National Health and Nutrition Examination Survey; U.S.= United States; y = years.

* Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements (mean n<30; 90th percentile n<80).

[a] Consumption in infants also includes intakes from hypoallergenic infant formula, which is not currently being considered as an intended use.

Table 3.4.2.2-2 Summary of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL from Infant Formula and Toddler Formula in the U.S. in Infant and Toddler Populations (2017-2018 NHANES Data)

Population Group	Age Group	*Per Capita* Intake (mg/kg bw/day)[a]		Consumer-Only Intake (mg/kg bw/day)[a]			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Infants	0 to 6 m	191	455	64.7	118	295	494
Infants	7 to <12 m	122	264	69.6	84	175	293
Toddlers	1 to 3 y	2*	NA	4.2	18	50*	113*

2'-FL = 2'-fucosyllactose; bw = body weight; m = months; n = sample size; NA = not applicable; NHANES = National Health and Nutrition Examination Survey; U.S. = United States; y = years.

* Indicates an intake estimate that may not be statistically reliable, as the sample size does not meet the minimum reporting requirements (mean n<30; 90th percentile n<80).

[a] Consumption in infants also includes intakes from hypoallergenic infant formula, which is not currently being considered as an intended use.

3.4.2.3 Comparison of the Estimated Daily Intake of 2'-FL from Proposed Conditions of Use (Infants) versus Mature Human Milk

The estimated daily intake of 2'-FL in infants from all proposed conditions of use (taken from Table 3.4.2.1-2) is compared to that from breast milk (taken from in Table 3.2-1) in Table 3.4.2.3-1, on a body weight basis. Mean consumer-only intakes of 2'-FL in infants from all proposed conditions of use, ranging between 355 and 520 mg/kg body weight/day, are within the average range of 2'-FL intakes from mature human milk of 197 to 770 mg/kg body weight/day, whereas 90th percentile intakes, ranging between 570 and 886 mg/kg body weight/day, are below the maximum estimated daily intake of 2'-FL from mature Secretor milk of 1,254 mg/kg body weight/day (see Table 3.4.2.3-1).

As indicated in Section 3.2, the use level of 2'-FL previously concluded to be GRAS in term (non-exempt) infant formula and toddler formula of 2.4 g/L corresponds to the representative mean concentration of 2'-FL in mature human milk (calculated in GRN 546). The estimated daily intake of 2'-FL from infant formulas and toddler formula (taken from Table 3.4.2.2-2) is compared to that from breast milk (taken from in Table 3.2-1) in Table 3.4.2.3-1, on a body weight basis. Mean consumer-only intakes of 2'-FL from infant formulas and toddler formula range between 175 and 295 mg/kg body weight/day and are within the average range of 2'-FL intakes from mature human milk of 197 to 770 mg/kg body weight/day. The 90th percentile intakes from infant formulas and toddler formula range from 293 to 494 mg/kg body weight/day, which is below maximum 2'-FL intakes from mature Secretor milk of 1,254 mg/kg body weight/day (see Table 3.4.2.3-1).

Table 3.4.2.3-1 Comparison of the Estimated Daily Per Kilogram Body Weight Intake of 2'-FL from All Proposed Conditions of Use, Infant Formula and Toddler Formula Only, and Human Milk

Population Group	Age Group	Consumer-Only Intake from All Proposed Uses (mg/kg bw/day)[a]		Consumer-Only Intake from Infant and Toddler Formulas (mg/kg bw/day)[a]		Intake from Mature Human Milk (mg/kg bw/day)		
		Mean	P90	Mean	P90	Mean (All; 2'-FL levels from LD6 to 100)	Mean Range (All; 2'-FL levels from LD 5 to 100)	Max (Secretors; 2'-FL levels from LD ≤30)
Infants	0 to 6 m	355	570	295	494	430	197 to 770	1,254
Infants	7 to <12 m	520	886	175	293			

2'-FL = 2'-fucosyllactose; bw = body weight; LD = Lactation Day; m = months; P90 = 90th percentile.
[a] Consumption in infants also includes intakes from hypoallergenic infant formula, which is not currently being considered as an intended use.

The intakes presented in the above scenarios do not take into account the possibility that a breastfed infant could consume complementary foods with 2'-FL. To assess the potential exposure, the concentration of 2'-FL in breast milk, the amount of breast milk consumed, and the intakes of 2'-FL from complementary foods must all be considered. As discussed in Section 3.2, concentrations of 2'-FL decrease as lactation progresses (Castanys-Muñoz *et al.*, 2013), with the highest observed concentrations occurring in Secretor milk within the first month of lactation (Gabrielli *et al.,* 2011; Galeotti *et al.,* 2012), a time when there is very little, if any, consumption of complementary foods. Thus, additive exposure to the highest estimate of background 2'-FL intake from breast milk is unlikely. Furthermore, as consumption of complementary foods increase, consumption of breast milk decreases, such that additive exposure will be occasional and transient. Therefore, it is highly unlikely that a breastfed infant would be both a high consumer of 2'-FL from breast milk and a high consumer of 2'-FL from complementary foods, and as such, no safety concerns are anticipated due to consumption of complementary foods supplemented with 2'-FL by breastfed infants.

3.4.3 Dietary Intake from Foods for Special Dietary Uses

Kyowa also intends to market 2'-FL for use in foods for special dietary uses, specifically, oral nutritional supplements and formula for enteral tube feeding. Oral nutritional supplements are intended for the general population (ages 2 and up). The recommended conditions of use are 2 g 2'-FL/45 g powdered serving or 250 mL ready to consume product, consumed twice per day for a total daily intake of 4 g 2'-FL/day. The use of 2'-FL in enteral tube feeding formula is intended for ages 11 and up and is proposed at a use level of 20 g/L in the final, ready to consume product. The recommended conditions of use for enteral tube feeding formula are 5 g 2'-FL per 250 mL, consumed twice per day, for a total intake of 10 g/day.

Foods for special dietary use containing 2'-FL are not intended to be consumed in combination with any other supplemental sources of 2'-FL and will be labeled as such. Consumption of 2'-FL from foods for special dietary use will be substitutional and not additive to consumption of 2'-FL from other sources.

3.4.4 Summary and Conclusions

Consumption data and information pertaining to the proposed food uses of 2'-FL were used to estimate the *per capita* and consumer-only intakes of this ingredient for specific demographic groups and for the total U.S. population (ages 2 years and older). Intake from use of 2'-FL in infant formulae and toddler formulas (intended for ages 1 to 3 years) only was also evaluated in infants and toddlers. There were a number of assumptions included in the assessment which render exposure estimates suitably conservative. For example, it was assumed in this exposure assessment that all food products within a food category contain 2'-FL at the maximum specified level of use. In reality, the levels added to specific foods will vary depending on the nature of the food product and it is unlikely that 2'-FL will have 100% market penetration in all identified food categories.

More than 72.1% of the population groups consisted of consumers of food products in which 2'-FL is proposed for use. Considering all proposed food uses, the resulting consumer-only mean and 90th percentile intakes of 2'-FL by the total U.S. population (≥2 years of age) were estimated to be 9.4 g/person/day (143 mg/kg body weight/day) and 17.4 g/person/day (298 mg/kg body weight/day), respectively. Among the individual population groups, the highest mean and 90th percentile intakes of 2'-FL on an absolute basis were determined to be 11.7 g/person/day (146 mg/kg body weight/day) and 20.6 g/person/day (261 mg/kg body weight/day), respectively, as identified among the elderly. Infants 0 to 6 months of age had the lowest consumer-only intakes on an absolute basis of 2.4 and 4.3 g/day at the mean and 90th percentile, respectively, while infants 7 to <12 months had the highest daily mean and 90th percentile intakes on a body weight basis, of up to 520 mg/kg body weight/day (4.7 g/person/day) and 886 mg/kg body weight/day (8.0 g/person/day), respectively. Top contributors to total mean intakes were: "Term infant formula" in infants 0 to 6 months of age (contributed 65% to total mean intakes); "Term infant formula" and "Other baby foods" in infants 7 to <12 months of age (contributed 20 and 25% to total mean intakes, respectively); and "Breads and baked goods" in all remaining population groups (contributed 28 to 57% to total mean intakes). The mean and 90th percentile consumer-only intakes of 2'-FL from use in infant formulas and toddler formula only were highest in infants 0 to 6 months of age on both an absolute and body weight basis, at 295 mg/kg body weight/day (1.87 g/person/day) and 494 mg/kg body weight/day (2.96 g/person/day), respectively.

The estimated daily intake of 2'-FL from proposed conditions of use in infants was compared to that from mature human milk. 2'-FL intakes are up to 3-fold higher when additive exposure from formula and conventional foods are considered together. Mean consumer-only intakes from all proposed uses (355 to 520 mg/kg body weight/day) are within the average range of 2'-FL intakes from mature breast milk (197 to 770 mg/kg body weight/day), whereas 90th percentile intakes (570 to 886 mg/kg body weight/day) are below the maximum estimated daily intake of 2'-FL from mature Secretor milk (1,254 mg/kg body weight/day). Considering exposure from infant formulas and toddler formula only, mean and 90th percentile consumer-only intakes of 2'-FL (up to 295 and 494 mg/kg body weight/day, respectively) are within the average range of 2'-FL intakes from mature human milk (197 to 770 mg/kg body weight/day), and below maximum 2'-FL intakes from mature Secretor milk (1,254 mg/kg body weight/day). As 2'-FL intakes from all proposed conditions of use are within background exposure from human milk in infants, a vulnerable population group, 2'-FL is considered to be safe for all population groups.

Breastfed infants are not expected to be high consumers of both 2'-FL from breast milk and 2'-FL from complementary foods, as the highest observed concentration of 2'-FL in breast milk occurred in Secretor milk within 1 month of lactation, at which point infants are unlikely to be exposed to complementary foods. Furthermore, the concentration of 2'-FL in human milk decreases as lactation progresses, and the consumption of breast milk would decrease as the consumption of complementary foods increases. Thus, additive exposure from high-level consumption of 2'-FL from breast milk and high-level consumption of complementary foods is unlikely. Therefore, no safety concerns are anticipated due to consumption of complementary foods supplemented with 2'-FL by breastfed infants.

Part 4. §170.240 Self-Limiting Levels of Use

No known self-limiting levels of use are associated with the use of 2'-FL.

Part 5. §170.245 Experience Based on Common Use in Food Before 1958

Not applicable.

Part 6. §170.250 Narrative and Safety Information

6.1 Introduction

The conclusion that 2'-FL produced by fermentation using a genetically modified strain of *E. coli* W is GRAS for use as an ingredient in non-exempt infant formula, conventional foods, and foods for special dietary uses is based on scientific procedures.

Kyowa's 2'-FL has been demonstrated by LC-MS, ^{1}H NMR, and ^{13}C NMR to be structurally and chemically identical to 2'-FL that is naturally present in human breast milk, and therefore, the natural background dietary exposure to 2'-FL from the consumption of human milk is the primary consideration in the assessment of the safety of Kyowa's 2'-FL. Background dietary exposure to 2'-FL was discussed in Section 3.2 and the mean intake of 2'-FL from mature human milk by infants was determined to range between 197 and 770 mg/kg body weight/day, with a maximum intake of up to 1,254 mg/kg body weight/day among infants from Secretor mothers. The estimated daily intake of 2'-FL from the proposed conditions of use was discussed in Section 3.4. Mean consumer-only intakes in infants from all proposed uses (355 to 520 mg/kg body weight/day) are within the average range of 2'-FL intakes from mature breast milk (197 to 770 mg/kg body weight/day), whereas 90th percentile intakes (570 to 886 mg/kg body weight/day) are below the maximum estimated daily intake of 2'-FL from mature Secretor milk (1,254 mg/kg body weight/day). Therefore, natural background dietary intakes of 2'-FL from the consumption of human milk support the safety of Kyowa's 2'-FL ingredient under the proposed conditions of use. As 2'-FL intakes from all proposed conditions of use are within background exposure from human milk in infants, a vulnerable population group, 2'-FL is considered to be safe for all population groups.

The composition of Kyowa's 2'-FL is similar to other 2'-FL ingredients previously concluded to be GRAS and notified to the U.S. FDA without questions. Specifications for Kyowa's 2'-FL produced by a genetically modified strain of *E. coli* W are compared to those for other 2'-FL ingredients previously concluded to be GRAS and notified to the U.S. FDA without questions (GRNs 546, 571, 650, 735, 749, 852, 897, and 929) in Table 6.1-1. As discussed in Section 2.3.1, the proposed purity specification for Kyowa's 2'-FL produced using a genetically modified strain of *E. coli* W is similar to the purity of 2'-FL produced using a genetically modified strain of *E. coli* K-12 (*i.e.*, ≥82% dry basis *vs.* ≥82% AUC; as specified in GRN 749) and lower than the purity specifications for other 2'-FL ingredients produced using genetically modified strains of *E. coli* BL21, *E. coli* K12, or from chemical synthesis (*i.e.*, ≥90 to ≥95% purity). Kyowa's limits for potential impurities of the production process, including D-lactose (≤5%), L-fucose (≤1%), difucosyllactose (≤3%), fucosylgalactose (≤3%), and D-glucose and D-galactose (≤1% combined) (all determined by HPLC-PAD) are similar to or lower than the specification limits for the same compounds in other 2'-FL ingredients (synthetic and microbial source) concluded to be GRAS and notified to the U.S. FDA (see Table 6.1-1 below). Lactose, fucose, and difucosyllactose are naturally-occurring components of human milk; fucosylgalactose is a naturally-occurring breakdown product of fucosylated oligosaccharides; galactose is a naturally-occurring breakdown product of lactose; and glucose is a naturally-occurring breakdown product of lactose, a common dietary component, and serves as a starting material for the biosynthesis of 2'-FL. The exposures to these carbohydrates from the intended uses of Kyowa's 2'-FL ingredient are expected to be insignificant compared to background exposures and are not expected to pose safety concerns.

Kyowa's 2'-FL ingredient is purified using similar processes as those previously reported to the U.S. FDA for the purification of 2'-FL. Purification processes described in other GRAS notices for 2'-FL generally involve several microfiltration, ultrafiltration, and/or nanofiltration steps to remove microbial biomass, proteins, DNA, lipopolysaccharides, minerals, and other small molecules (GRNs 546, 571, 650, 735, 749, 852, 897, and 929). The manufacturing process for Kyowa's 2'-FL similarly includes several microfiltration steps and an ultra-filtration step. The use of anionic/cationic resins to remove charged compounds (*e.g.,* proteins, DNA, organic acids, inorganic salts, and colored compounds) was noted in GRNs 571, 650, 735, 749, 852, 897, and 929 but not in GRN 546, and Kyowa uses a series of cationic resin and anionic resin ion exchangers for the same purposes. Electrodialysis also has been reported as a method of removal of charged molecules (GRNs 571, 650, 852, and 929). One or more treatments with activated charcoal or other adsorbent materials (activated carbon or adsorbent/polymeric resin) are used for the removal of colorants and other unspecified impurities (GRNs 546, 571, 650, 735, 749, 852, 897, and 929); Kyowa uses activated carbon for this purpose. In GRN 735, a pasteurization step is included late in the purification process as a further control against microbial contamination. In several of the GRAS notices, the purification of 2'-FL also includes a crystallization step. In this process, crystallized 2'-FL is washed with various solvents (acetic acid or ethanol) to remove any residual salt, carbohydrate, or biomolecule impurities prior to drying to produce the final 2'-FL ingredient (GRNs 650, 749, 852, and 897). Kyowa's 2'-FL is not purified by crystallization, similar to 2'-FL notified in GRNs 546, 571, 735, and 929.

Due to the compositional similarity between Kyowa's 2'-FL ingredient and other 2'-FL ingredients produced synthetically or by microbial fermentation, it was concluded that Kyowa's 2'-FL produced with a genetically modified strain of *E. coli* W is equivalent to 2'-FL produced by chemical synthesis or from other microbial sources and that safety data from previous GRAS Notices for 2'-FL are applicable to Kyowa's 2'-FL ingredient.

The safety of Kyowa's 2'-FL ingredient is also supported by the results of published preclinical toxicology and human studies conducted on other 2'-FL ingredients produced synthetically or by microbial fermentation and the conclusions of various experts qualified by scientific training and experience to evaluate the safety of food ingredients, including those used in infant formula, who have previously concluded that other 2'-FL ingredients were GRAS under conditions of use similar to those proposed in the current dossier (GRNs 546, 571, 650, 735, 749, 815, 852, 897, and 929). The U.S. FDA responded with no questions on the conclusions of GRAS status (U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a,b, 2020d, 2021). EFSA (2015) also has evaluated the safety of 2'-FL and concluded that it was safe under conditions of use similar to those proposed in the current GRAS notice. Safety data from GRNs 546, 571, 650, 735, 749, 815, 852, 897, and 929 are incorporated herein by reference and discussed briefly below in Sections 6.3 through 6.5. An updated literature search was conducted to identify any new published scientific information pertinent to the safety of 2'-FL published since the previous GRAS evaluations (see Section 6.2). No studies were identified that would contradict Kyowa's conclusion of GRAS status for 2'-FL. The identified studies are discussed in Sections 6.4.2 and 6.5.

Kyowa has conducted a battery of toxicological studies on their 2'-FL ingredient (Lot A; assay 92%) [Oguma, 2019a,b (unpublished); Tsuboi, 2020 (unpublished)]. Kyowa's 2'-FL ingredient was not mutagenic in a bacterial reverse mutation assay conducted in accordance with Good Laboratory Practice (GLP) and Organisation for Economic Co-operation and Development (OECD) Test Guideline 471 (*Bacterial reverse mutation test*) (OECD, 1997a, 1998; Oguma, 2019b [unpublished]). Kyowa's 2'-FL ingredient also was not genotoxic in an *in vivo* micronucleus study that was conducted in accordance with GLP and OECD 474 (*Mammalian erythrocyte micronucleus test*) (OECD, 1998, 2016a; Oguma, 2019a [unpublished]). In a 90-day toxicity study conducted in accordance with OECD 408 (*Repeated dose 90-day oral toxicity study in rodents*) and GLP (OECD, 1998, 2018), there were no mortalities, abnormal clinical signs, or toxicologically relevant compound-related adverse effects on any measured parameter, and the study authors determined the no-observed-adverse effect level (NOAEL) to be 2,000 mg/kg body weight/day, the highest dose tested (Tsuboi, 2020 [unpublished]). The results of these unpublished studies on Kyowa's 2'-FL ingredient corroborate the safety of the ingredient (see Section 6.4.1 for summaries).

The use of Kyowa's 2'-FL as an ingredient in enteral tube feeding formula at levels up to 20 g/L (for patients 11 years of age or older) is supported by the comprehensive body of safety data pertaining to 2'-FL in pre-clinical and human studies and the safety of poorly-digestible carbohydrates in general in enteral feeding at levels that exceed the recommended intake of 2'-FL from the intended use in formula for enteral tube feeding (see Section 6.5.3). Notably, the U.S. FDA responded with no questions to the GRAS status of 2'-FL under the conditions of use specified in GRN 897, including use in enteral tube feeding formula at levels up to 20 g/L (U.S. FDA, 2020b).

Finally, Kyowa's 2'-FL ingredient was concluded to be of low allergenic risk due to the effective removal of the production organism, residual DNA, and proteins; the lack of residual milk proteins; and the lack of published reports of sensitization, case reports of allergic reactions, or allergenicity studies on 2'-FL (see Section 6.6). The results of 2 studies in infants with confirmed or suspected cow's milk protein allergy and/or feeding intolerance in which 2'-FL ingredients were concluded to be safe and suitable in these populations support the lack of allergenicity of 2'-FL (see Section 6.5.1).

Table 6.1-1 Comparison of Kyowa's 2'-FL Specifications to Other 2'-FL Ingredients on the U.S. Market

Specification Parameter	2'-FL GRAS Notices Submitted to the U.S. FDA[a]							Kyowa's 2'-FL
	GRN 546	GRNs 571, 929	GRN 650	GRN 735	GRN 749	GRN 852	GRN 897	
Properties								
Source	Synthetic	Genetically modified strains of *E. coli* BL21	Genetically modified strains of *E. coli* K-12	Genetically modified strains of *E. coli* K-12	Genetically modified strains of *E. coli* K-12	Genetically modified strains of *E. coli* K-12	Genetically modified strain of *E. coli* K12	Genetically modified strains of *E. coli* W
Appearance	Powder	Powder or liquid concentrate	Powder or agglomerates	Homogenous powder	Powder	Powder or agglomerates	Powder	Powder
Color	White to off-white	White to ivory (powder); colorless to slightly yellow (concentrate)	White to off-white	White	White to off-white/ivory	White to off-white	White to off-white	White to off-white
Purity	≥95.0%	≥90%	≥94.0%	≥90%	≥82% (AUC)	≥90%	>96%	≥82% dwb
Water	≤9.0%	≤9.0% (powder only)	≤5.0%	≤5.0%	≤9.0%	≤9.0%	≤5.0%	≤9.0%
Ash	≤0.2%	≤0.5%	≤1.5%	≤0.2%	≤0.5%	≤1.5%	≤0.5%	≤0.5%
Residual protein	1,000 mg/kg	≤100 mg/kg	100 mg/kg	≤100 mg/kg	≤100 mg/kg	≤100 mg/kg	≤100 µg/g	≤10 mg/kg (0.001%)
pH	3.0 to 7.5 (20°C, 5% solution)	-	3.2 to 5.0 (20°C, 5% solution)	3.0 to 7.5 (10% solution)	-	3.2 to 7.5 (20°C, 5% solution)	-	4.0 to 9.0 (20°C, 5% solution)
Other Carbohydrates								
D-Lactose	-	≤5%	≤3.0%	≤3.0%	<8.0% (AUC)	≤3.0%	<5%	≤5.0%
L-Fucose	-	≤3%	≤1.0%	≤2.0%	See other CHOs	≤2.0%	-	≤1.0%
Glucose and Galactose	-	≤3%	-	≤2.0%	See other CHOs	-	-	≤1.0%
2'-Difucosyl-D-lactose	-	≤5%	≤1.0%	-	<7% (AUC)	≤2.0%	<5%	≤3.0%
2'-Fucosyl-D-lactulose	-	-	≤1.0%	-	See other CHOs	≤2.0%	-	-
3-Fucosyllactose	-	≤5%	-	-	See other CHOs	-	-	-
Fucosyl-galactose	-	≤3%	-	-	See other CHOs	-	-	≤3.0%
Allo-lactose	-	-	-	≤2.0%	-	-	-	-
Other CHOs	-	-	-	-	<6%[b] (AUC)	-	<5%	-

Table 6.1-1 Comparison of Kyowa's 2'-FL Specifications to Other 2'-FL Ingredients on the U.S. Market

Specification Parameter	2'-FL GRAS Notices Submitted to the U.S. FDA[a]							Kyowa's 2'-FL
	GRN 546	GRNs 571, 929	GRN 650	GRN 735	GRN 749	GRN 852	GRN 897	
Residual Solvents								
Acetic acid (as free acid and/or sodium acetate)	≤0.3%	-	≤1.0%	-	-	≤1.0%	-	-
Residual solvents	≤50 mg/kg singly; ≤200 mg/kg in combination	-	-	-	-	-	-	-
Heavy Metals								
Arsenic	-	≤0.2 mg/kg	-	≤0.1 mg/kg	≤0.2 mg/kg	≤0.1 mg/kg	≤0.2 mg/kg	≤0.2 mg/kg
Cadmium	-	≤0.1 mg/kg	-	≤0.01 mg/kg	≤0.05 mg/kg	≤0.05 mg/kg	≤0.05 mg/kg	≤0.2 mg/kg
Lead	≤0.8 mg/kg	≤0.02 mg/kg	≤0.1 mg/kg	≤0.05 mg/kg	≤0.05 mg/kg	≤0.05 mg/kg	≤0.05 mg/kg	≤0.2 mg/kg
Mercury	-	≤0.5 mg/kg	-	≤0.05 mg/kg	≤0.1 mg/kg	≤0.05 mg/kg	≤0.1 mg/kg	≤0.2 mg/kg
Iron	-	-	-	-	-	-	-	≤10 mg/kg
Aluminum	-	-	-	≤4.8 mg/kg	-	-	-	-
Microbial Parameters								
Aerobic plate count	≤500 CFU/g	≤10,000 CFU/g (powder) or ≤5,000 CFU/g (concentrate)	≤500 CFU/g	≤3,000 CFU/g	≤1,000 CFU/g	<500 CFU/g	<1,000 CFU/g	≤1,000 CFU/g
Molds	≤10 CFU/g	≤100 CFU/g (powder) or ≤50 CFU/g (concentrate)[c]	≤10 CFU/g	≤10 CFU/g	≤100 CFU/g	<100 CFU/g[c]	<100 CFU/g[c]	≤100 CFU/g
Yeasts	≤10 CFU/g	≤100 CFU/g (powder) or ≤50 CFU/g (concentrate)[c]	≤10 CFU/g	≤10 CFU/g	≤100 CFU/g	<100 CFU/g[c]	<100 CFU/g[c]	≤100 CFU/g
Salmonella	Absent in 25 g	Absent in 100 g (powder) or 200 mL (concentrate)	Absent in 25 g	Absent in 25 g	Absent in 100 g	Absent in 25 g	Absent in 750 g	Absent in 100 g

Table 6.1-1 Comparison of Kyowa's 2'-FL Specifications to Other 2'-FL Ingredients on the U.S. Market

Specification Parameter	2'-FL GRAS Notices Submitted to the U.S. FDA[a]							Kyowa's 2'-FL
	GRNs 546	GRNs 571, 929	GRN 650	GRN 735	GRN 749	GRN 852	GRN 897	
Enterobacteriaceae	Absent in 10 g	Absent in 11 g (powder) or 22 mL (concentrate)	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g	Absent in 10 g
Cronobacter spp.	Absent in 10 g	Absent in 100 g (powder) or 200 mL (concentrate)	Absent in 10 g	Absent in 25 g	Absent in 100 g	Absent in 10 g	Absent in 300 g	Absent in 100 g
Listeria monocytogenes	Absent in 25 g	-	Absent in 25 g	-	Absent in 25 g	Absent in 25 g	Absent in 25 g	Absent in 25 g
Bacillus cereus	≤50 CFU/g	-	≤50 CFU/g	≤100 CFU/g	≤10 CFU/g	-	<10 CFU/g	≤50 CFU/g
Residual endotoxins	≤50 EU/mg	≤300 EU/g (powder only)	-	≤10 EU/mg	≤300 EU/g	≤10 EU/mg	-	≤10 EU/mg
Escherichia coli	-	-	-	Absent in 10 g	-	-	-	-
Staphylococcus aureus	-	-	-	Absent in 1 g	-	-	<10 CFU/g	-
Sulfite reducing clostridia spores	-	-	-	≤30 CFU/g	-	-	<10 CFU/g	-
Clostridium perfringens	-	-	-	Absent in 1 g	-	-	<10 CFU/g	-
Enterococci	-	-	-	-	-	-	<100 CFU/g	-
Other Contaminants								
Aflatoxin M_1	-	≤0.025 µg/kg (powder only)	-	≤0.2 µg/kg	<0.025 µg/kg	-	-	-
Aflatoxin B_1	-	-	-	-	<1 µg/kg	-	-	-
Nitrite	-	-	-	≤1 mg/kg	-	-	-	-
Nitrate	-	-	-	≤50 mg/kg	-	-	-	-

- = not specified; 2'-FL = 2'-fucosyllactose; AUC = area under the curve; CFU = colony-forming units; CHOs = carbohydrates; dwb = dry weight basis; EU = endotoxin units; FDA = Food and Drug Administration; GRAS = Generally Recognized as Safe; GRN = GRAS Notice; U.S. = United States.

[a] Units were converted to match those from Kyowa Hakko's 2'-FL specifications, for direct comparison.

[b] Includes 3-fucosyllactose, 2-fucosyl-D-lactulose, fucosyl-galactose, glucose/galactose, fucose, sorbitol/galactitol, mannitol, trihexose.

[c] Yeasts and molds, together.

6.2 Literature Search

Kyowa considered the totality of publicly available data and information relevant to the safety of 2'-FL, and literature searches for studies relevant to the safety of 2'-FL were conducted. Comprehensive and detailed searches of the published scientific literature were conducted for studies published through 07 December 2021 using the electronic search tool, ProQuest Dialog™, with several databases, including Adis Clinical Trials Insight, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, and ToxFile®. Consistent with the requirements of the GRAS standard, conclusions on the GRAS status of 2'-FL have considered all publicly available sources of information including favorable and potentially unfavorable information. Based on Kyowa's search of the literature, the company is not aware of published studies to suggest 2'-FL is unsafe for use as a food ingredient.

6.3 Absorption, Distribution, Metabolism, and Elimination

The absorption, distribution, metabolism, and excretion of 2'-FL has been comprehensively reviewed in previous GRAS Notices of 2'-FL submitted to the U.S. FDA (GRNs 546, 571, 650, 735, 749, 815, 852, and 897) and in EFSA's Safety Opinions on 2'-FL and 2'-FL/DFL (EFSA, 2015, 2019)—the information is incorporated herein by reference. No additional studies describing the metabolic fate of 2'-FL were identified from the literature search. A brief summary of the metabolic fate of 2'-FL is provided below.

HMOs, including 2'-FL, are resistant to hydrolysis by digestive enzymes in the upper digestive tract (Engfer *et al.*, 2000; Gnoth *et al.*, 2000), and are either partially fermented by the intestinal microbiota or excreted unchanged in the feces (Brand-Miller *et al.*, 1995, 1998; Chaturvedi *et al.*, 2001b; Coppa *et al.*, 2001; Albrecht *et al.*, 2011; Kuntz *et al.*, 2019). 2'-FL fermentation products have been demonstrated to be absorbed into the systemic circulation and distributed to organs (liver, heart, spleen, kidney, and brain) of wild-type but not germ-free mice 2 hours following the oral administration of ^{13}C-labelled 2'-FL at a dose of 1 g/kg body weight/day, with highest levels observed 5 hours post-administration (Kuntz *et al.*, 2019). In the wild-type mice, ^{13}C was detected in urine but at lower levels than in feces, indicating that feces was the primary route of elimination. In contrast, no ^{13}C was detected in the urine of germ-free animals, indicating that the intestinal microbiota were required for the fermentation of 2'-FL. The results of this study support the previous conclusions that 2'-FL is not absorbed from the upper gastrointestinal tract, is fermented by the intestinal microbiota, and primarily excreted in the feces.

Neutral HMOs (such as 2'-FL) were demonstrated to be transported across the intestinal epithelium by receptor-mediated transcytosis and paracellular pathways in an *in vitro* model using heterogeneous human epithelial colorectal adenocarcinoma cells (Gnoth *et al.*, 2001). However, only low levels of 2'-FL have been detected in the urine and plasma of breastfed infants (Rudloff *et al.*, 1996, 2012; Obermeier *et al.*, 1999; Chaturvedi *et al.*, 2001b; Dotz *et al.*, 2014; Goehring *et al.*, 2014), infants receiving formula supplemented with 2'-FL (Marriage *et al.*, 2015), and rats administered a single oral dose of 2'-FL (Vazquez *et al.*, 2017), indicating that small amounts of 2'-FL are absorbed. 2'-FL absorption profiles were demonstrated to be similar in plasma samples collected from infants fed formulas supplemented with 2'-FL and infants fed human milk (Marriage *et al.*, 2015).

The levels of other carbohydrates in Kyowa's 2'-FL produced with a genetically modified strain of *E. coli* W are comparable to those in other 2'-FL ingredients (synthetic and microbial source) which have been concluded to be GRAS and notified to the U.S. FDA. These other carbohydrates (lactose, fucose, and difucosyllactose) are naturally occurring components of human milk or a human breakdown product of naturally-occurring fucosylated oligosaccharides (fucosylgalactose), while glucose and galactose are naturally-occurring breakdown products of lactose and are common dietary components. These other carbohydrates will follow endogenous routes of absorption, distribution, metabolism, and excretion (ADME). No safety concerns are expected due to the low dietary exposure to these carbohydrates from the intended uses of Kyowa's 2'-FL compared to background dietary exposures and their endogenous ADME profile.

6.4 Toxicological Studies

6.4.1 Studies of Kyowa's 2'-FL Ingredient

Kyowa has conducted a battery of toxicological studies on their 2'-FL ingredient (Lot A; assay 92%) [Oguma, 2019a,b (unpublished); Tsuboi, 2020 (unpublished)]. The results of these unpublished studies on Kyowa's 2'-FL ingredient corroborate the results of published toxicology studies on other 2'-FL ingredients and corroborate the safety of Kyowa's 2'-FL ingredient.

6.4.1.1 Genotoxicity

The potential mutagenicity of Kyowa's 2'-FL ingredient was evaluated in a bacterial reverse mutation assay conducted in accordance with GLP and OECD 471 (OECD, 1997a, 1998; Oguma, 2019b [unpublished]). In this study, *Salmonella* Typhimurium strains TA100, TA1535, TA98, and TA1537 and *E.coli* strain WP2 *uvrA* were incubated with Kyowa's 2'-FL (Lot A; assay 92%) at concentrations of 0 (water as negative control or positive controls[4]), 313, 625, 1,250, 2,500, or 5,000 µg/plate, in the presence or absence of metabolic activation. The assay was conducted according to the pre-incubation method. No precipitation of the test material or increase in the number of revertant colonies were observed in the presence or absence of metabolic activation, and the study authors concluded that 2'-FL was not mutagenic under the conditions of this study.

[4] Positive controls include: 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide; sodium azide; 2-methoxy-6-chloro-9-[3-(2-chloroethyl)aminopropylamino]acridine•2HCl; 2daminoanthracene; and benzo[a]pyrene.

The potential genotoxicity of Kyowa's 2'-FL ingredient (Lot A; assay 92%) was evaluated in an *in vivo* micronucleus study that was conducted in accordance with GLP and OECD 474 (OECD, 1998, 2016a; Oguma, 2019a [unpublished]). In this study, male Slc:ICR mice (5/group) were administered oral doses of 0, 500, 1,000, or 2,000 mg 2'-FL/kg body weight. Water was used as a negative control, while mitomycin C was used as a positive control. Two doses were administered 24 hours apart, and bone marrow samples were collected 22 to 24 hours after the final administration. There were no deaths and no abnormalities in the general condition of the mice. No statistically significant differences in the frequency of micronucleated immature erythrocytes or the proportion of immature erythrocytes were reported. The study was considered to be properly conducted as the frequency of the micronucleated immature erythrocytes in the negative and positive control groups were within the range of historical laboratory controls and there was a significant increase in micronucleated immature erythrocytes in the positive control group compared to the negative control group. The study authors concluded that 2'-FL does not induce chromosomal aberrations in mice.

6.4.1.2 Subchronic Toxicity

The potential toxicity of Kyowa's 2'-FL ingredient was evaluated in a 90-day toxicity study conducted in accordance with OECD 408 and GLP (OECD, 1998, 2018; Tsuboi, 2020 [unpublished]). In this study, 6-week-old Crl:CD(SD) rats (10/sex/group) were administered Kyowa's 2'-FL (Lot A; assay 92%) in distilled water by gavage at doses of 0 (distilled water), 500, 1,000, or 2,000 mg/kg body weight/day. The animals were observed daily throughout the study period, with body weight, food consumption, and behavioral observations made regularly. A functional observational battery was conducted during Week 12 of the study period, while ophthalmology and urinalysis were conducted during Week 13. Hematology, clinical biochemistry, organ weight, gross necropsy, and histopathology examinations were conducted upon study termination. Several statistically significant differences were reported with respect to food consumption, functional observation, clinical biochemistry, organ weights, gross necropsy, and histopathology. However, due to the small magnitudes of effect on these parameters, lack of dose-response relationship, and lack of correspondence between organ weight changes and gross or histopathological observations, these effects were considered by the study authors to be incidental and not toxicologically relevant.

Overall, no mortality, abnormal clinical signs, or toxicologically relevant compound-related adverse effects on any measured parameters were reported, and the study authors determined the NOAEL to be 2,000 mg/kg body weight/day, the highest dose tested.

6.4.2 Studies of Other 2'-FL Ingredients

6.4.2.1 Acute Toxicity Study

A single dose oral toxicity study was conducted with a 2'-FL ingredient manufactured by Kyowa and produced by fermentation using modified *E. coli* W production strains different from that described herein [BoZo Research Center Inc., 2016a (unpublished)]. The 2'-FL test article was reported to contain 95.3% 2'-FL (100% purity on a dry weight basis) and 4.9% water.

A single oral dose of 5,000 mg 2'-FL/kg body weight was administered to Sprague-Dawley rats (5/sex/group) at 6 weeks of age following an overnight fast (approximately 18 hours). Rats were observed for a 14-day period following administration (Day 0). General condition was evaluated at 5, 15, and 30 minutes, as well as 1, 2, 4, and 6 hours following administration, and daily throughout the rest of the observation period. Body weight was evaluated on Days 0, 1, 2, 7, 10, and 14. At the end of the 14-day observation period, organs, tissues, and body cavities were evaluated macroscopically. There were no clinical signs, gross pathological abnormalities, adverse effects on body weight, or mortalities reported throughout the study. The results of this unpublished study corroborate the results of published toxicology studies on other 2'-FL ingredients.

6.4.2.2 Subchronic Toxicity Studies

As discussed in Section 6.1, due to the compositional similarity between Kyowa's 2'-FL ingredient and other 2'-FL ingredients produced synthetically or by microbial fermentation, it was concluded that Kyowa's 2'-FL produced with a genetically modified strain of*E. coli* W is equivalent to 2'-FL produced by chemical synthesis or from other microbial sources and that safety data from previous GRAS Notices for 2'-FL are applicable to Kyowa's 2'-FL ingredient. Summaries of the identified published data relevant to safety are provided below.

Several repeat-dose toxicity studies assessing the safety and tolerability of 2'-FL produced synthetically and from microbial sources have been conducted in neonatal, juvenile, and adult rats (summarized in Table 6.4.2.2-1). Repeat-dose toxicity studies of 2'-FL/DFL produced by microbial fermentation (GRN 815 Glycom A/S) are also summarized in Table 6.4.2.2-1 for completeness as the ingredient is mostly composed of 2'-FL [82.5% (w/w) 2'-FL] [Flaxmer, 2017 (unpublished); Phipps *et al*., 2018]. These studies have been comprehensively reviewed in previous GRAS Notices of 2'-FL (summarized in Table 6.4.2.2-1) and evaluated by EFSA, and are thus incorporated herein by reference [Jennewein Biotechnologie, 2013, 2014a (unpublished); Coulet *et al*., 2014; Penard, 2015 (unpublished); Glycosyn, LLC and Friesland Campina Domo B.V., 2016, 2017a,b (unpublished) – in Glycosyn, LLC and Friesland Campina Domo B.V., 2018; van Berlo *et al*., 2018].

A 14-day repeat-dose toxicity study of 2'-FL in rats from Days 2 to 16 of life not evaluated in previous GRAS Notices or by EFSA was identified in the literature and is summarized in detail below (Azagra-Boronat *et al*., 2019). A recent 7-day oral tolerance study and a 13-week oral toxicity study of an HMO mixture containing 5 HMOs, 1 of which is 2'-FL, is also briefly summarized below (Parschat *et al*., 2020), as is a 90-day study of a product containing 31.5% 2'-FL and 59.4% lacto-*N*-fucopentaose I (LNFP-I) (Phipps *et al*., 2021).

Studies Included in Previous GRAS Notices for 2'-FL and Evaluated by EFSA

The lowest author-reported NOAEL of 2'-FL from 90-day oral toxicity rat studies is 5,000 mg/kg body weight/day [Coulet *et al*., 2014; Penard, 2015 (unpublished)] (see Table 6.4.2.2-1).

It should be noted that the EFSA Panel considers the NOAEL of 2'-FL from the study conducted by Coulet *et al*. (2014) to be 2,000 mg/kg body weight/day, the lowest dose tested, based on the following effects: (i) decreased relative kidney weight in the 2'-FL high-dose female group; (ii) the unexplained deaths of 1 male and 1 female in the high-dose 2'-FL group; and (iii) significant changes in the hematological and clinical blood parameters in the 2'-FL mid- and high-dose groups (EFSA, 2015).

Relative kidney weights were significantly decreased in females only from the high-dose 2'-FL group compared to controls and determined to be associated with minimal cortical tubular epithelial cytoplasmic vacuolation in the kidney in female rats from the mid- and high-dose 2'-FL groups and the fructooligosaccharide (FOS) reference high-dose control group at the end of the treatment period (Coulet *et al*., 2014). However, as histological findings were not reported in males, were not dose-dependent, and were not associated with any relevant clinical pathology changes or histological evidence of degeneration, the study authors concluded that the finding was not adverse. Furthermore, as the same renal histological finding was reported in control animals at the end of the recovery period, the study authors concluded that its origin is unclear. The study authors indicated that unexplained deaths were also reported in rats from the FOS reference high-dose control group, including 1 male on Day 12, 1 male on Day 13, and 1 female during the recovery period on Day 108. As the cause of death could not be determined by histopathological evaluation, the study authors concluded that a relationship to treatment could not be demonstrated. The study authors also indicated that changes in hematological and clinical blood parameters were minor, incidental, and of no biological or toxicological significance as individual values generally remained within the historical control ranges, lacked dose-response relationships, were often limited to 1 sex, generally also occurred in the FOS reference group, and were not supported by any other findings amongst other clinical parameters or histopathological observations.

In all other 90-day oral toxicity rat studies of 2'-FL (Jennewein Biotechnologie GmbH, 2015; Penard, 2015; van Berlo *et al*., 2018), these effects were not reported in dosing groups receiving greater than 2,000 mg 2'-FL/kg body weight/day. As mentioned above, these studies have been previously evaluated by independent Panels of qualified experts and the conclusions of GRAS status have been notified to the U.S. FDA without questions. According to GRN 815 (Section 6.3.2.1, page 44 of GRN 815), these experts unanimously concluded that the study authors' NOAEL determinations were appropriate (Glycom A/S, 2018).

Newly Identified Studies

In a newly identified study, 2'-FL produced by microbial fermentation (>90% purity; Danone Nutricia Research; production strain not reported) was administered daily by gavage for a duration of 14 days to 2-day-old Lewis rats (LEW/OrlRj) at a dose of 0 (water vehicle control) or 2,000 mg/kg body weight/day (Azagra-Boronat *et al*., 2019). Body weight and naso-anal and tail lengths were measured daily, and fecal samples were obtained to assess stool weight and consistency daily. Fecal samples were also obtained on Day 8 for the analysis of the microbiota composition. Tissue samples were obtained from 4 pups per dam from each group that were euthanized on Days 8 and 16 of life. Upon sacrifice on Days 8 and 16, organ weights (spleen, thymus, liver, small intestine, and large intestine) and the length of the small and large intestines were measured. Specific immune cell populations in mesenteric lymph nodes and plasma immunoglobins were assessed. The central part of the small intestine was used to quantify intestinal gene expression and a fragment of the distal jejunum was used to analyze histomorphometric changes, while the remaining parts of the small intestine were used to quantify cytokine release. Short-chain fatty acids (SCFAs acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, and heptanoic acids) were analyzed in the caecum at the end of the study period. Urine samples were obtained post-mortem for metabolomic analysis.

Animals given 2'-FL had a slight but significant increase in body weight on Day 16 compared to the control group. Body mass indices were comparable in all animals, but treated animals exhibited a greater body-to-tail-length ratio on Days 8 and 16. The study authors concluded that changes in growth were not biologically relevant. No significant difference was reported in organ weights except a decrease in the relative colonic weight on Day 16 in treated rats compared to controls, which was likely due to the non-biologically relevant increased body weight reported in this group. Stool weight and consistency were not affected by 2'-FL administration. The study authors concluded that 2'-FL was safe and well-tolerated as relative organ weights and stool characteristics were not affected.

No significant differences were reported for expression levels of genes involved with immunoglobulin (Ig) A secretion, toll-like receptors, cytokines, or epithelial barrier function proteins between the 2 groups. Intestinal villi height, villi area, and villus-to-crypt ratio increased significantly on Day 8 in treated rats compared to the control group, considered positive trophic effects of 2'-FL on intestinal growth. No significant difference was reported in microbial diversity within the fecal matter of rats. In the taxonomic phylum level, the proportion of Actinobacteria was lower, while that of Firmicutes was higher in the treated group compared to the control group. Certain butyrate-producing bacteria (*Roseburia*, *Ruminococcus*, and *Blautia*) were present only in rats supplemented with 2'-FL. With respect to SCFA levels, treated rats had significantly lower total SCFA levels which was mainly attributed to a decrease in the levels of acetic and propionic acids. However, the relative proportion of butyric acid was 2-fold greater in rats administered 2'-FL compared to the control group. Metabolic analysis of urine samples revealed that rats administered 2'-FL excreted significantly greater amounts of tricarboxylic acid cycle metabolites, metabolites from the nicotinate and nicotinamide pathway, gut microbial metabolites of choline, and metabolites of the oligosaccharide. Plasma concentrations of Ig, namely IgG2b, increased by 50% in 2'-FL-exposedrats on Day 8 compared to the control group, and levels of IgG2a, IgG2c, and IgA also increased significantly by Day 16. In mesenteric lymph nodes, a significant increase and decrease in the proportion of T cells and B cells, respectively, was reported. Cytokine levels were comparable in both groups on Day 16, but levels of almost all cytokines measured [interleukin (IL)-1β, IL-4, IL-6, IL-12, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α] decreased by 50% on Day 16 in the 2'-FL group. Based on the results of this study, the authors concluded that 2'-FL administered to pups at 2,000 mg/kg body weight/day was safe and well-tolerated, improved maturation of the immune system, and supported the development of a balanced gut microbiome.

The safety of HMO MIX I (Jennewein Biotechnologie, Rheinbreitbach, Germany) containing 5 HMOs, namely 2'-FL (47.1% dry weight), 3-fucosyllactose (16.0% dry weight), lacto-*N*-tetraose (23.7% dry weight), 3'-sialyllactose (4.1% dry weight), and 6'-sialyllactose (4.0% dry weight), was evaluated in a 7-day oral tolerance study and a 13-week oral toxicity study (Parschat *et al.*, 2020). As the independent effects of 2'-FL cannot be isolated, a brief summary is provided.

In the 7-day oral tolerance study, female CD/Crl:CD rats (n = 5/group) were fed diets containing 0 or 10% of the HMO MIX I, equivalent to 6.7 to 13.7 g HMO MIX I/kg body weight/day. Mortality, clinical signs, body weight, and food and water consumption were monitored daily. There were no changes in mortality, clinical signs (including fecal appearance and consistency) between groups, nor were there any treatment-related differences in body weight and food and water consumption. Therefore, a dose of 10% of the HMO MIX I was chosen for the 13-week oral toxicity study.

In the 13-week oral toxicity study, male and female CD/Crl:CD rats (n = 10/sex/group) were fed diets containing 0 or 10% of the HMO MIX I, equivalent to 5.67 and 6.97 g HMO MIX I/kg body weight/day in males and females, respectively. Corresponding 2'-FL intakes from the HMO MIX I were reported to be 2.67 and 3.28 g/kg body weight/day in males and females, respectively. Parameters evaluated included mortality, clinical signs, body weight, food and water consumption, neurological screening, ophthalmological and auditory examinations, clinical pathology, organ weights, and histopathology. The study authors reported no treatment-related effects and concluded that the NOAEL for the HMO MIX I was the only level tested (10% in the diet), equivalent to 5.67 and 6.97 g HMO MIX I/kg body weight/day for males and females, respectively.

A 90-day study of a product containing 31.5% 2'-FL and 59.4% LNFP-I (produced by Glycom *via* fermentation) was conducted to evaluate the safety of the product in neonatal rats (Phipps *et al*., 2021). The study was conducted in accordance with GLP and OECD 408 (OECD, 1998, 2018), with the exception that the animals were 7 days of age at the beginning of the dosing period as opposed to as soon as possible after weaning. This early dosing regimen was chosen to mimic early human development and to align with dosing periods developed for pediatric safety assessments. Neonatal Sprague-Dawley rat pups were allocated to receive 0 (water), 1,000, 3,000, or 5,000 mg/kg body weight/day by gavage for 90 days. The low- and mid-dose groups comprised 10 pups/sex/dose, while the control and high-dose groups comprised 15 pups/sex/dose. An additional group (15 pups/sex/dose) was administered 5,000 mg oligofructose/kg body weight/day for 90 days as a reference control. Recovery groups comprised 5 pups/sex/dose and were retained for a 4-week recovery period following administration of vehicle control, 5,000 mg oligofructose/kg body weight/day, or 5,000 mg 2'-FL/LNFP-I/kg body weight/day for 90 days.

Cage-side observations were conducted twice daily, while detailed physical examinations were conducted daily from Post-Natal Day (PND) 7 to 20 (*i.e*., the first 2 weeks of dosing) and weekly from PND 21. Body weights were measured daily from PND 7 to 20 and twice weekly thereafter. Food consumption was measured twice weekly from PND 21 onward. A functional observational battery was conducted during Week 11 of dosing, tests for auditory and visual function were conducted on PND 20, and tests for learning and memory were conducted during Week 12 of dosing. Blood samples were collected under fasted conditions at the end of the dosing and recovery periods and used for hematological and biochemical analyses. Urine samples also were collected at the end of the dosing and recovery periods. Organ weights were measured and gross pathology and ophthalmology examinations conducted upon necropsy, with histopathological examinations conducted on control, high-dose 2'-FL/LNFP-I, and decedent animals only.

No test item-related clinical signs were reported, nor were between-group differences in body weight, food consumption, or time to sexual maturity. Likewise, no adverse test item-related effects were reported with respect to behavior, estrous cycles, organ weights, or hematology, clinical biochemistry, or urinalysis parameters. No test item-related gross or histopathological abnormalities were reported. The study authors, therefore, concluded the NOAEL for this study to be 5,000 mg 2'-FL/LNFP-I/kg body weight/day (the highest dose tested, which provided 1,575 mg/kg body weight/day 2'-FL) and noted that the results support the use of these compounds in infant formula and foods for the general population.

Table 6.4.2.2-1 Summary of Subchronic and Chronic Toxicity Studies Conducted with 2'-FL

Species	Duration	Test Article	Dose (mg/kg bw/day)	Outcome Parameters	Conclusions on Safety	Results Relevant to GI Function and Tolerance	Reference
Rat, 26 to 28 days old, Crl:CD(SD) 5 F/group	7 days	Test Article 2'-FL produced using *Escherichia coli* BL21 [Jennewein Biotechnologie GmbH] Control Standard diet	0 or 10,000 (diet)[a]	Bw, food intake, stool consistency, mortality, clinical signs	No deaths and no changes in behavior, appearance, food consumption, or bw.	Stools were of normal consistency.	Jennewein Biotechnologie (2013) [unpublished] – In: Jennewein Biotechnologie GmbH (2015), Part 1
Rat, 7 days old, Wistar Crl:WI(Han) 5/sex/group	14 days	Test Article 2'-FL produced synthetically [Glycom A/S] Vehicle control Water Reference control FOS: 7,500 mg/kg bw/day	0, 2,000, 5,000, or 7,500 (gavage)	Mortality, clinical signs, bw, macroscopy	Well tolerated at the low-dose; limited effects at the mid-dose (transient lower bw gain and liquid feces); effects more marked at the high-dose (though similar effects were observed in the FOS group) and mortality in 2 of 5 females (cause of death not determined).	Liquid stools with occasional erythema in the urogenital area observed in rats from the mid- and high-dose groups and the FOS group – Days 1 to 3 and up to Days 9 to 11.	Coulet *et al.* (2014)
Rat, 6 to 7 weeks old, Wistar outbred (Crl:WI(Han)) 4 M/group	14 days	Test article 2'-FL produced using *Escherichia coli* K-12 [FrieslandCampina] Control cereal-based (closed formula) diet	0, 2,560, 5,080, or 7,990 (diet)	Clinical signs, bw, food intake, macroscopy, organ weights	No treatment-related effects with regard to clinical signs, bw, food intake, and macroscopic examination; decreased relative liver weights in the mid- and highdose groups (not considered toxicologically significant); increased absolute and relative cecal weights in the mid- and high-dose groups (likely from physiological adaptation to the test material).	NR	Glycosyn, LLC and Friesland Campina Domo B.V. (2016, 2017a,b) [unpublished] – in: Glycosyn, LLC and Friesland Campina Domo B.V. (2018)

Table 6.4.2.2-1 Summary of Subchronic and Chronic Toxicity Studies Conducted with 2'-FL

Species	Duration	Test Article	Dose (mg/kg bw/day)	Outcome Parameters	Conclusions on Safety	Results Relevant to GI Function and Tolerance	Reference
Rat, neonatal Crl:CD*(SD) 8/sex/group	14 days	Test Article 82.5% (w/w) 2'-FL and 9.7% (w/w) DFL mixture produced using *Escherichia coli* K-12 [Glycom A/S] Control Water	0, 4,000, or 5,000 (gavage)	Mortality, clinical signs, bw, gross macroscopic necropsy	One death reported on Day 15 in a male from the highd dose 2'-FL/DFL group, determined to be non-treatment related by the study authors; no biologically relevant differences in body weight between groups; no macroscopic abnormalities reported.	Red and/or yellow staining around the anus in some animals treated with 2'-FL/DFL, but these were transient (absent at the end of the observation period) and considered to be non-adverse.	Flaxmer, 2017 [unpublished]
Rat, 26 to 28 days old Crl:CD(SD) 10/sex/group	90 days	Test Article 2'-FL produced using *Escherichia coli* BL21 [Jennewein Biotechnologie GmbH] Control Standard diet	M 0 or 7,660 (diet) F 0 or 8,720 (diet)	Bw, bw gain, food intake, stool consistency, mortality, clinical signs, behavior, hematology, clinical biochemistry, urinalysis, ophthalmological examination, organ weights, histopathology	No substance-related adverse effects; the study authors reported NOAELs of 7,660 mg/kg bw/day for females and 8,720 mg/kg bw/daydor males.	Pale stools in 7 of 10 M and 4 of 10 F in the 2'-FL group (Days 9 to 69) – study authors concluded that this was due to undigested 2'-FL excreted in the feces.	Jennewein Biotechnologie (2014a) [unpublished] – In: Jennewein Biotechnologie GmbH (2015), Part 2

Table 6.4.2.2-1 Summary of Subchronic and Chronic Toxicity Studies Conducted with 2'-FL

Species	Duration	Test Article	Dose (mg/kg bw/day)	Outcome Parameters	Conclusions on Safety	Results Relevant to GI Function and Tolerance	Reference
Rat, juvenile Wistar Crl:WI(Han) 10/sex/group	90 days, followed by a 28-day recovery period (5/sex/group)[b]	Test article 2'-FL produced synthetically [Glycom A/S] Vehicle control Water Reference control FOS: 6,000 mg/kg bw/day	0, 2,000, 5,000 or 6,000 (gavage)	Mortality, clinical signs, ophthalmology, bw, food intake, hematology, coagulation, clinical chemistry, urinalysis, organ weights, and histopathology	The study authors reported a NOAEL of 5,000 mg/kg bw/day as any changes observed were determined to be of no biological or toxicological significance[c], whereas EFSA reported a NOAEL of 2,000 mg/kg bw/day based on the decrease in the relative kidney weight in the high-dose F group and significant changes in hematological parameters, unexplained deaths in 2 rats (1M, 1F) from the high-dose group, and changes in clinical blood parameters in the mid- and high-dose groups.	Diarrhea observed in a few rats from the low-dose group, and all rats from the mid- and high-dose groups and the FOS group (Day 1 until Days 12 to 13) – associated with erythema in the urogenital area of rats from the high-dose group and the FOS group.	Coulet *et al.* (2014)
Rat, 7 days old Crl:WI(Han) 10/sex/group	90 days, followed by a 28-day recovery period (5/sex/group)[b]	Test Article 2'-FL produced using *Escherichia coli* K-12 [Glycom A/S] Control NR Reference control FOS: 5,000 mg/kg bw/day	0, 2,000, 4,000, or 5,000 (gavage)	Mortality, bw, clinical signs, ophthalmology, hematology, coagulation, clinical chemistry, urinalysis, organ weights, and histopathology	The study authors reported a NOAEL of 5,000 mg/kg bw/day.	Liquid stools observed in rats from the mid- and high-dose groups (also observed in all rats from the FOS group).	Penard (2015) [unpublished]; taken from GRN 650 (Glycom A/S, 2016)

Table 6.4.2.2-1 Summary of Subchronic and Chronic Toxicity Studies Conducted with 2'-FL

Species	Duration	Test Article	Dose (mg/kg bw/day)	Outcome Parameters	Conclusions on Safety	Results Relevant to GI Function and Tolerance	Reference
Rat, 7 days old Crl:CD*(SD) 10/sex/group	90 days, followed by a 4-week recovery period (5/sex/group) [b]	Test Article 82.5% (w/w) 2'-FL and 9.7% (w/w) DFL mixture produced using *Escherichia coli* K-12 [Glycom A/S] Control Water Reference control FOS: 5,000 mg/kg bw/day	0, 1,000, 3,000, or 5,000 (gavage)	Mortality, clinical signs, ophthalmoscopy, bw, food consumption, sexual maturation, pre-weaning development, ulna growth, neurobehavior, hematology, coagulation, blood chemistry, urinalysis, organ weights, and histopathology	The study authors reported a NOAEL of 5,000 mg/kg bw/day.	NR	Phipps *et al.* (2018)
Rat, 7 days old SD 10/sex/group	90 days, followed by a 4-week recovery period (5/sex/group) [b]	Test Article 31.5% (w/w) 2'-FL and 59.4% (w/w) LNFP-I mixture produced using *Escherichia coli* K-12 [Glycom A/S] Control Water Reference control FOS: 5,000 mg/kg bw/day	0, 1,000, 3,000, or 5,000 (gavage)	Mortality, clinical signs, bw, food consumption, sexual maturation, neurobehavior, estrous cycle, hematology, blood chemistry, urinalysis, organ weight, histopathology	The study authors reported a NOAEL of 5,000 mg/kg bw/day.	NR	Phipps *et al.* (2021)

Table 6.4.2.2-1 Summary of Subchronic and Chronic Toxicity Studies Conducted with 2'-FL

Species	Duration	Test Article	Dose (mg/kg bw/day)	Outcome Parameters	Conclusions on Safety	Results Relevant to GI Function and Tolerance	Reference
Rat, Crl:WI(Han) 10/sex/group	90 days	Test article 2'-FL produced using *Escherichia coli* K-12 [FrieslandCampina] Control Cereal based rodent diet	0, 3, 6, or 10% (diet) M 0, 2170, 4270, or 7,250 mg/kg bw/day F 0, 2450, 5220, or 7760 mg/kg bw/day	Animal condition, mortality, behavior, motor activity, ophthalmoscopic observations, bw food and water consumption, hematology, clinical chemistry, urinalysis, organ weights, and histopathology	2'-FL did not induce adverse effects in any test group, the study authors reported a NOAEL of ≥7,250 mg/kg bw/day in M and ≥7,760 mg/kg bw/day in F.	NR	van Berlo *et al.* (2018)

2'-FL = 2'-fucosyllactose; bw = body weight; DFL = difucosyllactose; EFSA = European Food Safety Authority; F = females; FOS = fructooligosaccharides; GI = gastrointestinal; GRN = GRAS Notice; LNFP-I = lacto-*N*-fucopentaose I; M = males; NOAEL = no-observed-adverse-effect level; NR = not reported.

[a] Rats were administered 0 or 10% 2'-FL in the diet. Unit conversion was calculated using U.S. FDA (1993), assuming young rats.

[b] Control, FOS, and high-dose 2'-FL groups only.

[c] The authors noted that small statistically significant effects were observed in some of the hematological and clinical chemistry parameters, but that individual values generally remained within the historical control ranges, were without dose-response relationships, were often limited to 1 sex, and generally also occurred in the FOS reference group. Furthermore, the authors noted that none of the statistically significant changes were supported by findings from clinical parameters or histopathological observations.

6.4.2.3 Neonatal Piglet Study

The tolerability of 2'-FL was investigated in a GLP-compliant study in a neonatal piglet model (Hanlon and Thorsrud, 2014). This study was previously evaluated in GRN 571 (Jennewein Biotechnologie GmbH, 2015). Domestic Yorkshire Crossbred piglets (nt 27 males and 21 females) received liquid diets supplemented with 2'-FL at doses of 0, 200, 500, or 2,000 mg/L, from Lactation Day 2, for a duration of 3 weeks. The consumption of 2'-FL was calculated by the authors to correspond to doses of 0, 29.37, 72.22, and 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. An equal number of male and female piglets (6/sex) were allocated to the control and high dose groups, while 8 and 7 male piglets and 4 and 5 female piglets were allocated to the low and mid dose groups, respectively, to accommodate the imbalance in male and female piglets. The test article consisted of 2'-FL produced by fermentation from *E. coli* BL21 (manufactured by Jennewein Biotechonologie, Rheinbreitbach, Germany), and the vehicle consisted of commercially available Land O'Lakes® ProNurse® Specialty Milk replacement powder (Purina Animal Nutrition, Gray Summit, Missouri) dissolved at a concentration of 119.45 mg/mL in deionized water. Liquid diets (500 mL/kg/day) were offered to the piglets in a hand-filled bowl 6 times daily (3 hours± 15 minutes between each dose) for a minimum of 20 consecutive days. Piglets were housed individually in a controlled environment, with socialization between animals in the same group permitted in the mornings prior to feeding.

The animals were observed twice daily for morbidity, mortality, injury, and food availability. Body weight was recorded daily for the first week, and every other day for the remainder of the study. Detailed clinical examinations were conducted twice weekly. Blood samples were collected on Days 7 and 21 of the study (prior to daily feeding), and analyzed for changes in hematology parameters (leukocytes, erythrocytes, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, platelets, reticulocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and other cells), coagulation parameters (activated partial thromboplastin time, prothrombin time, and fibrinogen), and clinical chemistry (sodium, potassium, chloride, calcium, phosphorus, alkaline phosphatase, bilirubin, *gamma*-glutamyltransferase, aspartate aminotransferase, alanine aminotransferase, sorbitol dehydrogenase, urea nitrogen, creatine, total protein, albumin, triglycerides, cholesterol, and glucose). At the end of the study, the animals were terminated and subjected to gross necropsy. The brain, heart, kidney, large intestine, small intestine, and spleen were removed and weighed, and urine samples were collected for analysis of specific gravity, volume, and pH after necropsy. Additional microscopic examinations were conducted on all organs listed above, along with the eyes, optic nerve, gall bladder, stomach, gross lesions, lungs and bronchi, mesenteric lymph nodes, pancreas, and Peyer's patches.

No mortalities and no significant differences in mean body weight or food consumption were reported between groups throughout the consumption period. Watery feces were reported in 3 males and 2 females of the 2,000 mg/L group, 1 male and 2 females in the 500 mg/L group, and 2 males and 2 females in the 200 mg/L group (incidence in the control group not specified). However, the study authors concluded that this finding was not test-article related due to the lack of a dose-response and any other associated pathological findings. A lack of appetite was noted in 1 male and 2 females in the 2,000 mg/L group for 1 day, and 1 female in the 200 mg/L group for 2 days, but was not considered toxicologically relevant as there was no dose-response or significant change in growth or overall food intake. No compound-related effects on hematology, coagulation, or urinalysis were reported. Several statistically significant differences in clinical chemistry parameters were reported in administration groups compared to controls, the majority of which had no dose-response, occurred in 1 sex only, and were no longer significant at later time points of the study. Although increased alanine aminotransferase was reported in males in the 2,000 mg/L group on Days 7 and 21, the authors concluded that this finding was not compound-related as no differences in other clinical chemistry parameters for toxicity or absolute and relative liver weights were reported in this dose group, and no macroscopic or microscopic findings were observed.

Significant increases in absolute heart and kidney weights were reported in male piglets receiving 200 mg/L compared to controls, though there was no significant difference in relative organ weights between groups. In 1 male and 1 female in the 2,000 mg/L group and 1 female in the 500 mg/L group, mild to moderate inflammation (subacute to chronic) within the keratinized portion of the stratified squamous epithelium of the non-glandular stomach was reported. Focal loss and thinning of the keratinized portion of the stratified squamous epithelium was reported in the same male in the 2,000 mg/L group. Still, similar findings were not observed in the stomach and no ulceration was present. Furthermore, animals in all groups (including controls) had variable (minimal to mild) focal acute inflammation within the keratinized portion of the non-glandular stomach and variable thickness of the keratinized portion. Therefore, the study authors concluded that this finding cannot be definitively linked to the administration of 2'-FL as there was no clear dose-response, and these findings have been documented in historical controls (age and species-matched) from the same test facility.

Overall, the study authors concluded that dietary exposure to 2'-FL at concentrations of up to 2,000 mg/L, equivalent to up to 291.74 and 298.99 mg/kg body weight/day in males and females, respectively, is well-tolerated in neonatal piglets and did not cause adverse effects or affect growth.

6.4.2.4 Genotoxicity Studies

The genotoxic potential of 2'-FL, produced synthetically or by fermentation from *E.tcoli* K-12 and BL21, has previously been evaluated in various genotoxicity assays [Coulet *et al*., 2014; Jennewein Biotechnologie, 2014b,c (unpublished) In: Jennewein Biotechnologie, 2015 (part 1); Verspeek-Rip, 2015 (unpublished); Verbaan, 2015 (unpublished); Phipps *et al*., 2018, 2021; van Berlo *et al*., 2018]. In all instances, genotoxicity assays were conducted according to test guidelines from the OECD, including the bacterial reverse mutation test (OECD 471; OECD, 1997a), the *in vitro* mammalian cell gene mutation test [OECD 476 (*In vitro mammalian cell gene mutation tests using the Hprt and xprt genes*); OECD, 1997b], the *in vitro* mammalian micronucleus test [OECD 487 (*In vitro mammalian cell micronucleus test*); OECD, 2016b], and the *in vivo* mammalian micronucleus test (OECD 474; OECD, 2016a). These studies have been evaluated in previous GRAS evaluations submitted to the U.S. FDA without questions and are summarized in Table 6.4.2.4-1. An additional study not included in a previous GRAS assessment for 2'-FL submitted to the U.S. FDA was also included in this table (Phipps *et al*., 2021). In all studies, 2'-FL was concluded to be non-mutagenic and non-genotoxic under assay conditions. EFSA evaluated genotoxicity studies of synthetic 2'-FL (Coulet *et al.*, 2014), and concluded that the ingredient does not raise safety concerns with regard to genotoxicity (EFSA, 2015).

The mutagenicity of another 2'-FL ingredient (purity 95.3% w/w; water 4.9% w/w) manufactured by Kyowa and produced by modified strains of *E. coli* W (different than the production strain that is the subject of the current GRAS assessment) was evaluated in a bacterial reverse mutation assay [BoZo Research Center Inc., 2016b (unpublished)]. The assay was conducted according to OECD 471 (OECD, 1997a). Reverse mutation was assessed in various strains of *S.* Typhimurium (TA100, TA1535, TA98, and TA1537) and int*E. coli* WP2 *uvrA*, with and without metabolic activation, using the pre-incubation method. In the main test, 5 concentrations of 2'-FL were tested: 313, 625, 1,250, 2,500, and 5,000 µg/plate. There was no significant difference in the number of revertant colonies (defined as an increase of 2-fold or more) between the 2'-FL treatment groups and the negative control (distilled water), although there was a significant difference in the number of revertant colonies between the positive controls and the negative control. Thus, the study authors concluded that 2'-FL was non-mutagenic under the conditions of the assay. The results of this study corroborate the lack of mutagenicity reported in published studies of 2'-FL ingredients.

The mutagenicity and genotoxicity of Jennewein Biotechnologie's HMO MIX I (containing 47.1% 2'-FL on a dry weight basis) was evaluated in a bacterial reverse mutation and an *in vitro* micronucleus assay (Parschat *et al*., 2020). The methods and results are summarized in Table 6.4.2.4-1. The study authors concluded that the HMO MIX I is non-mutagenic and non-genotoxic under the conditions of the assays.

Table 6.4.2.4-1 Summary of Genotoxicity Studies of 2'-FL

Ingredient, Source (Manufacturer; Purity)	Test	Compliance	Test System/ Animal Species	Concentration/Dose	Controls	Results	Conclusion	Reference
Studies Reviewed in Previous GRAS Evaluations of 2'-FL and Notified to the U.S. FDA								
2'-FL, synthetic (Glycom; 99%)	Bacterial reverse mutation assay	OECD Principles of GLP (OECD, 1998); OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537	Plate incorporation assay 52, 164, 512, 1,600, or 5,000 µg/plate (+/- S9) Pre-incubation assays 492, 878, 1,568, 2,800, or 5,000 µg/plate (+/- S9)	Negative control Water (vehicle) Positive controls + S9: 2-aminoanthracene - S9: 2-nitrofluorene (TA98), sodium azide (TA100, TA1535), 9-aminoacridine (TA1537), t-butylhydroperoxide (TA102)	No cytotoxicity or precipitation; no biologically significant increase in the number of revertant colonies compared to the negative control.	Non-mutagenic under the conditions of the assay.	Coulet *et al.* (2014); GRN 546
	In vitro mammalian cell gene mutation assay	OECD Principles of GLP (OECD, 1998); OECD TG 476 (OECD, 1997b)	Cultured mouse lymphoma L5178Y cells	4 h exposure 492, 878, 1,568, 2,800, or 5,000 µg/mL (+/- S9) 24 h exposure 1.7, 5.4, 17, 52, 164, 512, 1,600, or 5,000 µg/mL (- S9)	Negative control Untreated medium Positive controls + S9: cyclophosphamide - S9: methylmethanesulfonate	No cytotoxicity or precipitation; no biologically relevant increases in mutant frequency with or without S9.	Non-genotoxic in mouse lymphoma cells under the conditions of the assay.	

Table 6.4.2.4-1 Summary of Genotoxicity Studies of 2'-FL

Ingredient, Source (Manufacturer; Purity)	Test	Compliance	Test System/ Animal Species	Concentration/Dose	Controls	Results	Conclusion	Reference
2'-FL from *Escherichia coli* BL21 (Jennewein; 92.4%)	Bacterial reverse mutation assay	GLP according to Directive 2004/9/EC (EC, 2004); OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537	Plate incorporation and the pre-incubation assays 0, 31.6, 100, 316, 1,000, 3,160, or 5,000 µg/plate (+/- S9)	Negative control DMSO (vehicle) Positive controls + S9: sodium azide (TA1535, TA100), 2-nitrofluorene (TA98), 9-aminoacridine (TA1537), or mitomycin C (TA102) - S9: 2-aminoanthracene (TA1535, TA100), or benzo(a)pyrene (TA98, TA102, TA1537)	No cytotoxicity; no increase in the number of revertant colonies compared to the negative control at any dose level with or without metabolic activation.	Non-mutagenic under the conditions of the assay.	Jennewein Biotechnologie (2014b) [unpublished] – In: Jennewein Biotechnologie (2015) GmbH [part 1]; GRN 571
	In vivo mammalian micronucleus assay	GLP according to Directive 2004/9/EC (EC, 2004); OECD TG 474 (OECD, 2016a)	Rat (*Rattus norvegicus*) / CD Crl:CD(SD) 5/sex/group	24 h exposure 0, 500, 1,000, or 2,000 mg/kg bw (single dose) 48 h exposure 0 or 2,000 mg/kg bw (single dose) Single dose (gavage) Bone marrow collected 24 h (all groups) or 48 h (negative control and high dose groups only) post-administration	Negative control 0.8% aqueous hydroxypropylmethyl-cellulose (vehicle) Positive control 27 mg/kg bw cyclophosphamide with 0.9% NaCl solution	No systemic toxicity; no increase in the incidence of micro-nucleated PCEs compared to the negative control.	Non-genotoxic under the conditions of the assay.	Jennewein Biotechnologie (2014c) [unpublished] – In: Jennewein Biotechnologie GmbH (2015) [part 1]; GRN 571

Table 6.4.2.4-1 Summary of Genotoxicity Studies of 2'-FL

Ingredient, Source (Manufacturer; Purity)	Test	Compliance	Test System/ Animal Species	Concentration/Dose	Controls	Results	Conclusion	Reference
2'-FL from *E.coli* K-12 (Glycom; 97.6%)	Bacterial reverse mutation assay	OECD Principles of GLP (OECD, 1998); OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537; *Escherichia coli* strain WP2uvrA	Plate incorporation assay 0, 52, 164, 512, 1,600, or 5,000 µg/plate (+/- S9) Pre-incubation assay 0, 492, 878, 1,568, 2,800, or 5,000 µg/plate (+/- S9)	Negative control Water (vehicle) Positive controls + S9: 2-nitrofluorene (TA98, TA1537, pre-incubation assay), methylmethanesulfonate (TA100), sodium azide (TA1535), ICR-191 (TA1537, plate incorporation assay), 9-aminoacridine (TA1537), or 4-nitroquinoline n-oxide (WP2uvrA) - S9: 2-aminoathracene	No cytotoxicity or precipitation; no biologically significant increase in the number of revertant colonies compared to the negative control.	Non-mutagenic under the conditions of the assay.	Verspeek-Rip (2015) [unpublished]; GRN 650
	In vitro micronucleus assay	OECD Principles of GLP (OECD, 1998); OECD TG 487 (OECD, 2014)	Cultured peripheral human lymphocytes	3 h exposure 512, 1,600, or 2,000 µg/mL (+/- S9) 24 h exposure 512, 1,600, or 2,000 µg/mL (- S9)	Negative control Water (vehicle) Positive controls + S9: cyclophosphamide - S9: mitomycin C	No cytotoxicity or precipitation; no statistically or biologically significant increases in the frequency of mono- or bi-nucleated cells with micronuclei in cells treated with 2'-FL compared to the negative control.	Non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.	Verbaan (2015) [unpublished]; GRN 650

Table 6.4.2.4-1 Summary of Genotoxicity Studies of 2'-FL

Ingredient, Source (Manufacturer; Purity)	Test	Compliance	Test System/ Animal Species	Concentration/Dose	Controls	Results	Conclusion	Reference
2'-FL from *E.coli* K-12 (Glycosyn, LLC and Friesland Campina Domo B.V.; 94%)	Bacterial reverse mutation assay	OECD Principles of GLP (OECD, 1998); OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537; *Escherichia coli* strain WP2uvrA	Plate incorporation assay 0, 62, 195, 556, 1667, or 5,000 µg/plate (+/- S9)	Negative control Phosphate buffered saline (vehicle) Positive controls + S9: 2-aminoathracene (TA98, TA100, TA1535, WP2 uvrA), or benzo(a)pyrene (TA1537) S9: sodium azide (TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98), sodium azide (TA100), or N-ethyl-N-nitrosourea (WP2uvrA)	No cytotoxicity; no biologically significant increase in the number of revertant colonies compared with the negative control.	Non-mutagenic under the conditions of the assay.	van Berlo *et al.* (2018); GRN 735
	In vitro micronucleus assay	OECD Principles of GLP (OECD, 1998); OECD TG 487 (OECD, 2014)	Cultured peripheral human lymphocytes	4 h exposure 0, 500, 1,000, or 2,000 µg/mL (+/- S9) 24 h exposure 0, 500, 1,000, or 2,000 µg/mL (- S9)	Negative control Culture medium Positive controls + S9: cyclophosphamide - S9: vinblastine	No cytotoxicity; no statistically or biologically significant increase in the frequency of MNBCs in cells treated with 2'-FL compared to the negative control.	Non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.	

Table 6.4.2.4-1 Summary of Genotoxicity Studies of 2'-FL

Ingredient, Source (Manufacturer; Purity)	Test	Compliance	Test System/ Animal Species	Concentration/Dose	Controls	Results	Conclusion	Reference
2'-FL/DFL *E. coli* K-12 [Glycom; 82.5% (w/w) 2'-FL; 9.7% (w/w) DFL]	Bacterial reverse mutation assay	OECD Principles of GLP (OECD, 1998); OECD TG 471 (OECD, 1997a)	*Solmonello typhimurium* strains TA98, TA100, TA1535, and TA1537; *Escherichia coli* strain WP2uvrA (pKM101)	Plate incorporation assay 0, 5, 15, 50, 150, 500, 1,500, or 5,000 µg/plate (+/- S9) Pre-incubation assay 0, 50, 150, 500, 1,500, or 5,000 µg/plate (+/- S9)	Negative control Water (vehicle) Positive controls + S9: 2-aminoanthracene (TA100, TA1535, WP2uvrA) or benzo[a]pyrene (TA98, TA1537) - S9: sodium azide (TA100, TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98) or 4-nitroquinoline-1-oxide (WP2uvrA)	No biologically relevant increase in the number of revertant colonies compared to the vehicle control.	Non-mutagenic under the conditions of the assay.	Phipps *et al.* (2018); GRN 815
	In vitro micronucleus assay	OECD Principles of GLP (OECD, 1998); OECD TG 487 (OECD, 2016b)	Cultured peripheral human lymphocytes	3 h exposure 0, 500, 1,000, or 2,000 µg/mL (+/- S9) 24 h exposure 0, 500, 1,000, or 2,000 µg/mL (- S9)	Negative control Water (vehicle) Positive controls + S9: cyclophosphamide - S9: colchicine and mitomycin C	No cytotoxicity; No significant increase in the frequency of MNBCs in cells treated with 2'-FL compared to the negative control.	Non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.	

Table 6.4.2.4-1 Summary of Genotoxicity Studies of 2'-FL

Ingredient, Source (Manufacturer; Purity)	Test	Compliance	Test System/ Animal Species	Concentration/Dose	Controls	Results	Conclusion	Reference
HMO MIX I [47.1% dw 2'-FL; 16.0% dw 3'-FL, 23.7% dw LNT; 4.1% dw 3'-SL; 4.0% dw 6'-SL] (Jennenwein Biotechnologie)	Bacterial reverse mutation assay	OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537	Plate incorporation assay 0, 5, 10, 31.6, 100, 316, or 600 mg/plate (+/- S9) Pre-incubation assay 0, 5, 10, 31.6, 100, 316, or 600 mg/plate (+/- S9)	Negative control Water (vehicle) Positive controls + S9: 2-aminoanthracene (TA100, TA1535) or benzo[a]pyrene (TA98, TA102, TA1537) - S9: sodium azide (TA100, TA1535), 9-aminoacridine (TA1537), 2-nitrofluorene (TA98), or mitomycin C (TA102)	No cytotoxicity or mutagenicity compared to the vehicle control.	Non-mutagenic under the conditions of the assay.	Parschat *et al.* (2020)
	In vitro micronucleus assay	OECD TG 487 (OECD, 2016b)	Cultured peripheral human lymphocytes	4 h exposure 0, 7.5, 15, 30, or 60 mg/mL (+/- S9) 24 h exposure 0, 7.5, 15, 30, or 60 mg/mL (- S9)	Negative control Water (vehicle) Positive controls + S9: cyclophosphamide - S9: colchicine and mitomycin C	No indications of chromosomal damage; frequency of micronucleate cells within the historical control range for the test item and vehicle controls.	Non-genotoxic under the conditions of the assay.	

Table 6.4.2.4-1 Summary of Genotoxicity Studies of 2'-FL

Ingredient, Source (Manufacturer; Purity)	Test	Compliance	Test System/ Animal Species	Concentration/Dose	Controls	Results	Conclusion	Reference
Studies Not Included in Previous GRAS Evaluations Submitted to the U.S. FDA								
2'-FL (31.5%), LNFP-I (59.4%), other carbohydrates (4.6%) (Glycom A/S)	Bacterial reverse mutation assay	OECD TG 471 (OECD, 1997a)	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537; *Escherichia coli* WP2 *uvr*A	Plate incorporation assay: 0, 5, 15, 50, 150, 500, 1,500, or 5,000 µg/plate (+/- S9) Pre-incubation assay: 0, 50, 150, 500, 1,500, or 5,000 µg/plate (+/- S9)	Negative control Water Positive controls + S9: benzo[a]pyrene and 2-aminoanthracene - S9: sodium azide, 9-aminoacridine, 2-nitrofluorene, and 4-nitroquinoline-1-oxide	No cytotoxicity or mutagenicity compared to the vehicle control.	Non-mutagenic under the conditions of the assay.	Phipps *et al.* (2021)
	In vitro micronucleus assay	OECD TG 487 (OECD, 2016b)	Cultured peripheral human lymphocytes	3-hour exposure: 0, 0.5, 5, 50, 500, 1,000, or 2,000 µg/mL (+/- S9) Additional 3-hour exposure: 500, 1,000, or 2,000 µg/mL (+ S9) 20-hour exposure: 0, 0.5, 5, 50, 500, 1,000, or 2,000 µg/mL (- S9)	Negative control Water Positive controls - S9: mitomycin C and colchicine + S9: cyclophosphamide	No indications of chromosomal damage; frequency of micronucleate cells within the historical control range for the test item and vehicle controls.	Not clastogenic or aneugenic under the conditions of the assay.	

+ S9 = with metabolic activation; - S9 = without metabolic activation; 2'-FL = 2'-fucosyllactose; 3'-FL = 3'-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; bw = body weight; DFL = difucosyllactose; DMSO = dimethyl sulfoxide; dw = dry weight; FDA = Food and Drug Administration; GLP = Good Laboratory Practice; GRN = GRAS Notice; h = hours; HMO = human milk oligosaccharide; LNFP-I = lacto-*N*-fucopentaose I; LNT = lacto-*N*-tetraose; MNBC = micronucleated binucleated cell; OECD = Organisation for Economic Co-operation and Development; PCE = polychromatic erythrocyte; TG = Test Guideline; U.S. = United States.

6.5 Human Studies

6.5.1 Intervention Studies

The safety and tolerability of 2'-FL supplementation, either alone or in combination with other oligosaccharides, has been evaluated in various clinical studies conducted in infants (Marriage *et al.*, 2015; Goehring *et al*., 2016; Kajzer *et al*., 2016; Puccio *et al*., 2017; Storm *et al*., 2019) and in a single clinical study conducted in adults (Elison *et al*., 2016) that were reviewed during previous evaluations of the GRAS status of 2'-FL ingredients. Clinical studies reviewed as part of GRAS Notices of 2'-FL submitted to the U.S. FDA are summarized below in Table 6.5.1-1, whereby it was consistently concluded by the study authors that supplementation of infant formula with 2'-FL at levels ranging from 0.2 to 1.0 g/L (with or without other HMOs) is safe and well-tolerated in infants, and that daily supplementation of up to 20 g 2'-FL is safe and well-tolerated in adults.

Table 6.5.1-1 Human Studies of 2'-FL Reviewed in Previous GRAS Evaluations Submitted to the U.S. FDA

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
Studies in Infants – Infant Formula							
131 full term, singleton infants, 0 to 8 days old MC, R, DB, C, PRO Country NR	1 month	Test group (ITT: n=46; PP: n=41) scFOS + 2'-FL C (ITT: n=42; PP n=36) No HMOs Matrix Milk-based formula Reference control group (ITT: n=43; PP: n=42) HM	2 g/L scFOS + 0.2 g/L 2'-FL	Primary Average MRSC from stool records enrollment to 35 DOL (PP population only) Secondary Formula intake, stool consistency, stool frequency, anthropometrics, percent feedings with spit-up/vomit	• NSD in MRSC, stool consistency, formula intake, feeding frequency, anthropometric data, or percent feedings with spit-up/vomit between groups. • SS increase in average number of stools per day in the HM group compared to all other groups from enrollment to 35 DOL (P<0.0001).	The study authors concluded that "*formula containing 2'-FL and scFOS was well tolerated in young infants as evidenced by stool consistency, formula intake, anthropometric data and percent feedings with spit-up/vomit similar to that of infants fed formula without oligosaccharides or HM*".	Kajzer *et al.* (2016)
420 healthy, full-term, singleton, infants, 0 to 5 days old R,DB,C,PRO U.S.	4 months	Test group (ITT: n=104 for low-dose or 109 for high dose; PP: n=70 for low-dose and 72 for high-dose) Synthetic 2'-FL + GOS C (ITT: n=101; PP: n=79) GOS only Matrix Milk-based formula Reference control group (ITT: n=106; PP: n=83) HM	0, 0.2, or 1.0 g/L 2'-FL in combination with 2.4, 2.2, 1.4 g/L GOS, respectively.	Primary Weight gain from DOL 14 to 119 Secondary Other anthropometric measures (weight, length, or head circumference), formula intake, tolerance [incidence of spitting-up and vomiting, and stool characteristics (frequency, consistency, and color)], and AEs	• NSD in mean weight gain between groups from DOL 14 to 119 or anthropometric measures. • SS increase in weight gain in HM group compared to low-dose 2'-FL group from DOL 14 to 28 (P=0.016); SS increase in weight gain in high-dose 2'-FL group compared to HM group from DOL 84 to 119 (P=0.022). • SS increase in formula intake in control group compared to low-dose 2'-FL group from enrollment to DOL 28 (P=0.024).	The study authors concluded that "*overall, there were no safety concerns noted*" for infant formulas containing HMOs and that "*formulas supplemented with 2'-FL are well tolerated*".	Marriage *et al.* (2015)

Table 6.5.1-1 Human Studies of 2'-FL Reviewed in Previous GRAS Evaluations Submitted to the U.S. FDA

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
					• SS increase in stool frequency in HM group compared to other groups from enrollment to DOL 28 (P<0.0001) and for 3-day periods before DOL 42 and 84 (P<0.0001 and 0.004, respectively); SS increase in stool frequency in HM group compared to control group for 3-day period before DOL 119 (P=0.008). • SS increase in percent feedings with spitting-up or vomit in all formula-fed groups compared to HM group from enrollment to DOL 28 (P≤0.05). • SS increase in MRSC in high-dose 2'-FL group compared to HM group from enrollment to DOL 28 (P=0.021); SS increase in MRSC in all formula groups compared to HM group at DOL 42, 84, and 119 (P≤0.009). • NSD in percentage of subjects with AEs or serious AEs between formula-fed groups.		

Table 6.5.1-1 Human Studies of 2'-FL Reviewed in Previous GRAS Evaluations Submitted to the U.S. FDA

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
420 healthy, full-term, singleton, infants, 0 to 5 days old R,dDB,dC,dPRO U.S.	4 months	Test group (n=37 for both the low- and high-dose groups, from which blood samples were analyzed) Synthetic 2'-FL + GOS C (n=39, from which blood samples were analyzed) GOS only Matrix Milk-based formula Reference control group (n=42 from which blood samples were analyzed) Breastfed infants	0, 0.2, or 1.0 g/L 2'-FL in combination with 2.4, 2.2, 1.4 g/L GOS, respectively	Levels of plasma inflammatory cytokines, *ex vivo* cytokine production by PBMCs, circulating lymphocyte populations in PBMCs, and immune cell proliferation and cell cycle, RSV NS1 viral load	• SS increase in circulating plasma concentrations of inflammatory cytokines IL-1a, ILdLb, IL-6, and TNF-α and antidnflammatory IL-1ra in control group compared to HM group (P≤0.05). • SS increase in production of TNF-α and IFN-γ by *ex vivo* RSV-induced PBMCs in control group compared to HM group (P≤0.05). • SS decrease in percentage of circulating T lymphocytes for total T cell and CD8+ in control group compared to HM group (P≤0.05; almost SS for CD4+, P=0.06). • SS decrease in CD8+ cells in high-dose 2'-FL group compared to HM group (P≤0.05). • SS increase in percentage of CD20+ cells in S phase in activated PBMCs in control group compared to HM group (P≤0.05). • SS increase in percentage of $CD8^{Lo}CD4$-cells in the G2/M phase in HM group compared to high-dose 2'-FL group (P≤0.05). • NSD in RSV NS1 viral load between groups.	The study authors concluded that "*infants fed formula supplemented with 2'-FL exhibit lower plasma and ex vivo inflammatory cytokine profiles, similar to those of a breastfed reference group*".	Goehring *et al*. (2016); same population as Marriage *et al*. (2015)

Table 6.5.1-1 Human Studies of 2'-FL Reviewed in Previous GRAS Evaluations Submitted to the U.S. FDA

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
175 healthy, full-term, formula-fed[a] infants, 0 to 14 days old MC, R, DB, C, P Italy (n=80) and Belgium (n=95)	6 months	Test group (ITT: n=88; PP: n=71 at 4 months) Synthetic 2'-FL + LNnT Supplied by Glycom A/S C (ITT: n=87; PP: n=75 at 4 months) No HMO Matrix Intact protein cow's milk-based infant formula	0 or 1.0 g/L 2'-FL + 0.5 g/L LNnT	Primary Weight gain through 4 months Secondary Other anthropometric measures (weight, length, BMI, head circumference, and corresponding z-scores), digestive tolerance (flatulence, spitting-up, and vomiting), digestive symptoms, stool characteristics (stool consistency and frequency), 76behavior patterns (restlessness, colic, and nighttime awakenings), formula intake, and morbidity (AEs and concomitant medications)	• NSD in mean weight gain between groups (PP and ITT populations). • NSD in anthropometric measures. • SS softer stools in test group compared to control group at 2 months (P=0.021; tended to be softer at 1 month: P=0.064). • SS decrease in frequency of colic in test group compared to control group at 4 months in infants delivered by cesarean section (P=0.035). • SS decrease in frequency of nighttime awakenings in test group compared to control group at 2 months (P=0.036). • Only 1 infant experienced an AE (cow's milk-protein allergy) considered to be related to the study formula; at 4 months, percentage of infants with >1 AE (test: 84.1%; control 90.8%), and >1 serious AE (test: 6.8%; control 11.5%), was similar between groups.	The study authors concluded that "*Infant formula with 2' FL and LNnT is safe, well-tolerated, and supports age-appropriate growth*". In EFSA's evaluation, it was noted that "*no difference in growth in infants who consumed a formula added with the combination of 2'-FL and LNnT (at the concentrations tested in the study), compared with the control formula infants, and that the growth curves were comparable to the WHO standard curves*". The Panel noted that "*the results on stool and microbiota endpoints do not raise safety concerns*"	Puccio *et al.* (2017)

Table 6.5.1-1 Human Studies of 2'-FL Reviewed in Previous GRAS Evaluations Submitted to the U.S. FDA

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
78 healthy, full-term, singleton, formula-fed[b] infants, 14 ± 5 days old MC, R, DB, C, P U.S.	6 weeks	Test group (ITT: n=38; PP: n=30) 2'-FL (not further specified) C (ITT: n=40; PP: n=33) No 2'-FL Matrix Whey protein-based partially hydrolyzed, infant formula with probiotic *B. lactis*	0 or 0.25 g/L[c]	Primary IGSQ scores Secondary Formula intake, tolerance (stool frequency, stool consistency, ease of passing, frequency of vomiting and spitting up, duration of crying and fussing), AEs, and anthropometric parameters	• NSD in mean IGSQ scores between groups at BL or EOT in PP and ITT populations. • Infants receiving test formula reported to have soft or loose stool 77% of the time; however, no significant difference in stool consistency was noted between groups. • SS increase in difficulty passing stools in control group compared to test group (P=0.04). • SS increase in number of infants who spit up >5 times/day in test group compared to controls (P-value not reported). • No difference in number of infants/group with spitting up reported as an AE (1/group). • Total of 72 AEs reported, with an equal number (36) split between groups; no serious AEs reported.	The study authors concluded that "*overall, there were no safety concerns noted*" and that "*the addition of 0.25 g/L of the HMO 2'-FL and probiotic B lactis is tolerated well based on a comprehensive tolerance assessment tool*".	Storm *et al.* (2019)
Studies in Adults							
100 healthy adults, 19 to 57 years of age R, DB, PC, P Denmark	2 weeks, following a 1- to 2-week run-in period	Test groups (n=30 per test group, 10 per dose) 1. Synthetic 2'-FL 2. Synthetic LNnT 3. Synthetic 2'-FL + LNnT (2:1 mass ratio, mixed)	5, 10, or 20 g/day	Anthropometric measures (heart rate and blood pressure), GI symptoms (assessed by GSRS questionnaire), AEs, stool frequency and consistency, clinical chemistry, hematology, abundance of gut microbiota	• No clinically significant difference in anthropometric measures, clinical chemistry parameters, or hematology parameters between groups.	The study authors concluded that "*2'-FL and LNnT are safe and well tolerated in healthy adults*". In their evaluation, the EFSA Panel noted that GI symptoms were significantly increased in individuals consuming 20 g 2'-FL/day compared to	Elison *et al.* (2016)

Table 6.5.1-1 Human Studies of 2'-FL Reviewed in Previous GRAS Evaluations Submitted to the U.S. FDA

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference
		Supplied by Glycom A/S PC (n=10) 2 g glucose Matrix Beverage (powder containing the test substance was dissolved in water)			• Total of 56 AEs reported by 44 subjects, all of which were judged as 'mild'; AEs usually reported as multiple symptoms (flatulence, bloating and constipation), primarily at EOT, and by subjects in high-dose groups. • SS increase in GSRS scores in high-dose 2'-FL group compared to control (nausea, rumbling, bloating, passing of gas, diarrhea, loose stools and urgency to pass stools), and mid- and high-dose LNnT groups compared to placebo (passing of gas), though overall scores remained low. • Some small but significant changes in stool frequency and consistency, as well as fecal biomarkers, but considered clinically irrelevant. • SS positive correlation between the concentration of 2'-FL and LNnT and increased Actinobacteria (P<0·05, R2= 28%); SS increased *Bifidobacterium* abundance in mid-dose 2'-FL group, all LNnT groups, and mid- and high-dose 2'-FL + LNnT group compared to control (P<0.05).	placebo, but that GI symptoms were not observed in individuals consuming 10 g 2'-FL/day.	

Table 6.5.1-1 Human Studies of 2'-FL Reviewed in Previous GRAS Evaluations Submitted to the U.S. FDA

Study Population, Design, Country	Duration	Groups	Dose	Safety-Related Outcome Parameters	Findings	Conclusions on Safety	Reference

2'-FL = 2'-fucosyllactose; AEs = adverse events; BL= baseline; BMI = body mass index; C = controlled; DB = double-blind; DOL = day of life; EFSA = European Food Safety Authority; EOT= end-of-treatment; FDA= Food and Drug Administration; GI = gastrointestinal; GOS= galactooligosaccharides; GRAS= Generally Recognized as Safe; GSRS = Gastrointestinal Symptom Rating Scale; HM= human milk; HMO= human milk oligosaccharide; IFN = interferon; IGSQ = Infant Gastrointestinal Symptom Questionnaire; IL= interleukin; ITT = intention-to-treat; LNnT = lacto-*N*-neotetraose; MC= multi-center; MRSC = mean rank stool consistency; NR = not reported; NSD = no significant difference; P = parallel; PBMC = peripheral blood mononuclear cell; PC= controlled; PP= per protocol; PRO= prospective; R = randomized; RSV= respiratory syncytial virus; scFOS = short-chain fructooligosaccharides; SS = statistically significant; TNF = tumor necrosis factor; U.S.= United States.

[a] Exclusively formula-fed at time of enrollment.

[b] Infants had to have been exclusively formula-fed for at least 3 days prior to enrollment.

[c] Lactose, corn maltodextrin (70/30), was used to maintain the amount of carbohydrates in both formulas at 11.2 g/100 kcal (absolute amount in each formula NR).

Newly Identified Studies

Studies of 2'-FL conducted in infants and adults that were identified in a comprehensive search of the published scientific literature conducted through 07 December 2021, and not included in previous GRAS evaluations submitted to the U.S. FDA or evaluated by EFSA, are described below.

Studies in Infants

Vandenplas *et al*. (2020) conducted a randomized, double-blind, placebo-controlled, multi-country study to evaluate the effects of a partially fermented infant formula on growth, safety, and tolerability. Subjects included healthy, full-term, European, exclusively formula-fed infants ≤14 days of age at baseline. Exclusively breastfed infants were included as a reference group. The test and control formulas were nutritionally complete cow's milk-based formulas, with the test formula supplemented with 26% fermented formula, 2'-FL (1 g/L; source not reported), short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) (9:1; 8 g/L), and milk fat (49.8% of total fat). The test and control formulas were administered from ≤14 days of age until 17 weeks of age, with anthropometric measures, gastrointestinal symptoms, and safety assessed monthly. No statistically significant differences were reported between the control and test groups with respect to growth, adverse events, serious adverse events, or gastrointestinal tolerability, and the authors concluded that the test formula supports adequate growth and is safe and well-tolerated in healthy term infants.

Roman *et al*. (2020) conducted an open-label, prospective study to evaluate the effects of infant formula containing 2'-FL and lacto-*N*-neotetraose (LNnT) (source not reported) on growth and tolerability. Study subjects included healthy, Spanish, full-term infants 7 days to 2 months of age at baseline, who were either exclusively breastfed (n=45), exclusively formula-fed (n=66), or fed with breast milk and infant formula (n=45). The partially-hydrolyzed, whey-based formula was provided *ad libitum* for 8 weeks, contained 1 g 2'-FL/L and 0.5 g LNnT/L, and was supplemented with *Lactobacillus reuteri* (concentration not reported). Anthropometric parameters were measured at baseline and end-of-treatment, while gastrointestinal symptoms were evaluated using the validated Infant Gastrointestinal Symptom Questionnaire at baseline and at the end of study Weeks 4 and 8. Adverse events were monitored by the subjects' caregivers throughout the study. Three adverse events were considered to be potentially product-related, and included 2 instances of cow's milk intolerance (1 in each of the exclusively formula-fed and breast/formula-fed groups) and 1 instance of irritability (in the exclusively formula-fed group). Six serious adverse events occurred (4 and 2 instances in the exclusively formula-fed and breast/formula-fed groups, respectively), all of which were bronchiolitis and considered unrelated to the study product. Overall, the incidence of adverse events was low and not significantly different between groups. The authors reported no significant between-group differences with respect to growth or gastrointestinal tolerability, and concluded that these results support the effectiveness, safety, and tolerability of the feeding regimes evaluated.

Storm *et al*. (2019) conducted a randomized, double-blind, placebo-controlled, multi-center study to assess the tolerability of a whey-based partially hydrolyzed infant formula containing *Bifidobacterium lactis* and 2'-FL (source not reported). Healthy, full-term, singleton, exclusively formula-fed infants 14 ± 5 days of age at baseline were given formula containing *B. lactis* with 0 or 0.25 g 2'-FL/L (n=40 and 38 infants, respectively) for 6 weeks. Tolerability was assessed primarily using the Infant Gastrointestinal Symptom Questionnaire, which included questions related to stool frequency, stool consistency, ease of passing, frequency of vomiting and spitting up, and duration of crying and fussing. Other measured parameters included formula intake, adverse events, and anthropometric parameters. Soft or loose stool was reported 77% of the time in infants given the 2'-FL formula, and a significant increase was reported in the number of infants who spit up >5 times/day in the 2'-FL formula group compared to the control group. A total of 72 adverse events were reported, which were split equally between the control and 2'-FL groups, with no serious adverse events reported. The study authors concluded that *"overall, there were no safety concerns noted"* and that *"the addition of 0.25 g/L of the HMO 2'-FL and probiotic B lactis is tolerated well based on a comprehensive tolerance assessment tool"*.

Leung *et al*. (2020) conducted a randomized, double-blind, placebo-controlled study to evaluate the effects of "young child formulas" containing bioactive proteins (immunoglobulins, lactoferrin, and transforming growth factor-β), 2'-FL (source not reported), and/or milk fat on the incidence of upper respiratory or gastrointestinal infections. Chinese children 1 to 2.5 years of age (n=114/group) were provided for 6 months with the control formula (standard milk formula) or test formulas containing bioactive proteins, 2'-FL, and milk fat at levels typically found in human breast milk (doses not reported); milk formula containing lower levels of bioactive proteins (doses not reported); or milk formula supplemented with 2'-FL (3 g/L; source of 2'-FL not reported) only. The study products were consumed in two 200-mL servings/day. Adverse events and stool frequency and consistency were recorded daily in a diary by the subjects' caregivers. Physical examinations were conducted by study personnel at clinic visits every 2 months during the study period. The authors noted that subjects consuming the formula containing 2'-FL only had longer (but not more frequent) upper respiratory tract infections, more incidences of cough and runny nose, and more days with fever compared to the control formula. Subjects consuming formula with bioactive proteins, milk fat, and 2'-FL had more gastrointestinal infection episodes than control subjects. However, the authors reported that there were no between-group differences in the incidence of adverse events or serious adverse events, with none of the reported events considered to be product-related. No between-group differences in growth were reported, and the authors concluded that the study formulas were safe and supported normal growth in toddlers.

Studies in Infants with Food Allergy and/or Feeding Intolerance

In addition to intervention studies in healthy term infants, studies in term infants with cow's milk protein allergy (CMPA), suspected food protein allergy, persistent feeding intolerance, or other conditions warranting the use of extensively hydrolyzed infant formula, were identified and are summarized below. The results of these studies support the safety of 2'-FL in sensitive infant populations (*i.e.*, those with food allergies or feeding intolerance).

An acute study conducted to assess the allergenicity, tolerability, and safety of a whey-based extensively hydrolyzed formula (EHF) supplemented with 2'-FL and LNnT at concentrations of 1.0 and 0.5 g/L, respectively, was assessed in infants with CMPA (Nowak-Wegrzyn *et al*., 2019). This study was previously included in a GRAS evaluation of 2'-FL (GRN 897 – DuPont Nutrition and Health, 2019). Healthy infants and children 2 months to 4 years of age with documented CMPA following a strict cow's milk protein-free elimination diet prior to enrollment were recruited from 12 U.S. study sites. Allergenicity was evaluated in a

cross-over double-blind placebo-controlled food challenge (DBPCFC). The sample size was calculated to meet the American Academy of Pediatrics (AAP) criteria for assessing hypoallergenicity of infant formulas, where, at minimum, it must be demonstrated with 95% confidence that 90% [95% lower bound confidence interval (CI) >90%] of infants with documented CMPA will not react with defined symptoms (AAP, 2000). The placebo control formula was a commercially available hypoallergenic EHF without HMO (Althéra®, Nestlé Health Science, Vevey, Switzerland). Study subjects were randomized to receive the first food challenge (test or placebo control EHF) 3 to 28 days after enrollment, and the second food challenge (alternate EHF) was administered 2 to 7 days after the first challenge. The food challenge was administered following a 1-hour fast. Following an initial lip dose challenge, oral doses of the assigned EHF were administered at 10- to 15-minute intervals (infants ≤1 year of age: 5, 10, 20, 30, 30, 35, and 50 mL for a total volume 180 mL; infants >1 year of age: 5, 10, 25, 45, 45, 45, and 65 for a total volume of 240 mL). The consumption of a minimum of 100 mL of EHF was considered evaluable. Subjects were observed for a 1-hour period during which allergic signs or symptoms (cutaneous, gastrointestinal, respiratory, or cardiovascular) determined to be related to the food challenge were documented on a standardized DBPCFC data collection form and assessed according to pre-defined pass/fail criteria. If no allergenicity was observed following DBPCFC to the test and control formulas, subjects were exposed to the test formula (240 mL/day) for an additional 7 to 9 days, as recommended by the AAP, to confirm the absence of a delayed allergic reaction. Tolerability and safety were also assessed during the 1-week open challenge through the recording of the following clinical parameters: daily stool frequency, color, consistency, and odor; frequency of flatulence; frequency of spitting-up and/or vomiting; allergic symptoms; adverse or serious adverse events.

The study authors determined that 61 subjects were required in the per protocol (PP) population, which would allow 2 reactions in the test group while still meeting the AAP criteria for hypoallergenicity (95% lower bound CI >90%). Overall, 67 subjects were randomized to receive the test or control formula in the first DBPCFC [intention-to-treat (ITT) population], 64 subjects completed the first DBPCFC (modified ITT population), and 61 subjects completed both the first and second DBPCFC (PP population). Of the modified ITT population, 63 of 64 subjects tolerated the test formula [98.4%; 95% CI lower bound 92.8%] and 61 of 62 subjects tolerated the control formula (98.4%; 95% CI lower bound 92.6%). Of the PP population 60 of 61 subjects tolerated the test formula (98.4%; 95% lower bound CI 92.5%)—the result was the same for the control formula. A total of 55 subjects completed the 1-week open challenge. No treatment-related gastrointestinal symptoms (flatulence, abnormal stool frequency/consistency, reactions, increased spitting-up or vomiting), reactions requiring early discontinuation, or serious adverse events were reported. The study authors concluded that the EHF supplemented with 2'-FL and LNnT was confirmed to be hypoallergenic according to AAP criteria and is suitable for the management of CMPA in infants and young children.

Ramirez-Farias *et al*. (2021) conducted a Good Clinical Practice (GCP)-compliant multicenter study in 47 infants (0 to 60 days of age) with suspected food protein allergy, persistent feeding intolerance, or other conditions warranting the use of extensively hydrolyzed infant formula. All infants were administered formula containing 2'-FL (0 or 0.2 g/L; source not reported) for 2 months, with 36 of the 48 enrolled infants completing the study. Measures of growth as well as daily formula intake, stool observations, and adverse events were recorded throughout the study. No adverse effects were reported with respect to growth or between-group differences in the incidence of adverse effects, and the study authors concluded that the formula containing 2'-FL was well tolerated and safe.

Studies in Adults

Iribarren *et al*. (2020) conducted a randomized, double-blind, placebo-controlled study to evaluate the effects of a 4:1 mixture of 2'-FL and LNnT (Glycom A/S) on fecal microbiota composition and gastrointestinal symptom severity in adults with irritable bowel syndrome (IBS). Subjects included 60 Swedish adults (40 women and 20 men) with IBS of moderate severity diagnosed according to the Rome IV criteria (IBS Symptom Severity Scale Score ≥175). Subjects were assigned to consume a glucose placebo or 5 or 10 g 2'-FL/LNnT/day for 4 weeks, followed by a 4-week follow-up period. IBS symptom severity, bowel habits, anxiety, and depression were assessed at baseline and at the end of Study Weeks 4 and 8. Two subjects withdrew from the study due to worsening of IBS symptoms, including 1 subject from each of the placebo and 10 g/day groups. No adverse effects on fecal microbiota were reported, and no between-group differences in severity of overall or individual gastrointestinal symptoms or symptom deterioration were reported. The study authors concluded that the study products were well tolerated and that gastrointestinal symptoms in adult IBS patients were not aggravated by consumption of 10 g of a 4:1 mixture of 2'-FL and LNnT/day for 4 weeks.

Ryan *et al.* (2021) conducted a single-arm, open label study to evaluate the effects of a nutritional formula containing 2'-FL in 20 adults with IBS, ulcerative colitis (UC), or celiac disease. Subjects were provided with 40 g of nutritional formula containing 2 g 2'-FL in additional to micronutrients and macronutrients, to be dissolved in 8 to 10 ounces of chilled water or juice and consumed twice daily for 6 weeks (total intake of 4 g 2'-FL/day). The Gastrointestinal Quality of Life Index (GIQLI), Digestive Symptom Frequency Questionnaire (DSFQ), and Inflammatory Bowel Disease Questionnaire (IBDQ) were administered at baseline and at the end of the consumption period. Stool samples were assessed for colony-forming units (CFU) of commensal bacteria and stool SCFAs at baseline and at the end of Week 6. Eight subjects withdrew from the study (2 prior to commencing the study intervention, 3 who were lost to follow up, and 3 due to a worsening of pre-existing gastrointestinal symptoms, gastrointestinal upset, or an unrelated viral infection). No compound-related adverse effects were reported with respect to the GIQLI, DSFQ, and IBDQ scores. Significant increases were reported in stool counts of *Faecalibacterium prausnitzii*, *Anaerotruncus colihominis*, and *Pseudoflavonifractor* species, as well as fecal levels of butyrate, acetate, and total SCFAs. Bifidogenic and butyrogenic effects were reviewed but no significant associations were reported. The study authors concluded that the consumption of 4 g 2'-FL/day in a nutritional formula had no adverse effects on the IBS and UC patient's gastrointestinal symptoms and the consumption was associated with an improvement in intra- and extra-intestinal symptoms.

6.5.2 Observational Studies

Health effects of 2'-FL in infants from mothers with high *versus* low levels of 2'-FL in breast milk have also been evaluated in prospective observational cohort studies. The relationship between HMO composition in maternal milk and infant growth (primarily weight) was evaluated in 6 studies (Alderete *et al.*, 2015; Sprenger *et al.*, 2017; Berger *et al.*, 2019; Larsson *et al.*, 2019; Lagström *et al.*, 2020; Binia *et al.*, 2021), and the relationship between HMO composition in maternal milk and infant body composition was further investigated in 3 of the 6 studies (Alderete *et al.*, 2015; Larsson *et al.*, 2019; Binia *et al.*, 2021). The purpose of these studies was to determine whether HMO composition influences excessive weight gain that has been reported in some breastfed infants, as HMOs modulate the gut microbiota, and gut microbiota potentially play a role in energy harvest and storage from the diet. In addition, 1 study was identified in which the relationship between maternal secretor status and episodes of infant diarrhea was evaluated (Muthumuni *et al.*, 2021). The studies are discussed in greater detail below.

Relationship Between HMO Composition in Maternal Milk and Infant Growth

The association between HMO composition in maternal milk and infant weight at 1 and 6 months of age was evaluated by Berger *et al*. (2019) in 156 Hispanic mother-infant pairs. HMO composition was analyzed by HPLC (type of detection not reported). Infant weight was measured at 1 and 6 months of age (no further details) and used to calculate weight-for-age z-scores (WAZ). Associations between HMO concentrations in maternal milk and WAZ was assessed by multiple linear regression adjusting for maternal age, pre-pregnancy body mass index (BMI), infant age, sex, and birth weight. No significant association between the concentration of 2'-FL in maternal milk and WAZ was observed at 1 or 6 months (significant association reported for lacto-*N*-fucopentaose only).

In an exploratory study (no power analysis), Sprenger *et al.* (2017) evaluated the effect of 2'-FL milk levels on infant growth in 50 healthy mother-infant pairs. As part of the inclusion criteria, mothers had to be willing to breastfeed at least up to 4 months, from Singapore who gave birth to term, singleton, infants (25 males and 25 females). Milk samples of approximately 30 mL were collected using a milk pump 30, 60, and 120 days postpartum from full expression from 1 breast while the baby was fed on the other breast, with an effort to collect a complete feed that included fore-, mid-, and hind-milk. The following HMOs were analyzed in milk samples in duplicate by liquid chromatography followed by high-performance anion exchange chromatography (HPAEC) coupled to a pulsed amperometry detector: 2'-FL, lacto-*N*-tetraose (LNT), LNnT, 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL). Outcomes evaluated related to infant growth included body weight, body length, body mass index, and head circumference.

2'-FL concentrations measured in breast milk samples collected 30 days postpartum were used to group mother-infant pairs into high (n=34; mean 2,170 mg/L; 95% CI of mean 1,880 to 2,460 mg/L) and low 2'-FL groups (n=16; mean 27 mg/L; 95% CI of mean 12 to 42 mg/L). Baseline characteristics and anthropometric findings at birth were similar between groups. Throughout the 4-month lactation period, 2'-FL and LNnT concentrations were significantly higher in milk samples from the high 2'-FL group compared to those from the low 2'-FL group. Contrary, LNT was the most abundant HMO in milk samples from the low 2'-FL group, and LNT concentrations were significantly higher in milk samples from the low 2'-FL group compared to those from the high 2'-FL group. Based on World Health Organization growth curves for infants and z-scores, no significant differences in growth parameters were observed over the 4-month study period. However, the study authors caution that the study was not powered to assess infant growth and relied solely on anthropometric endpoints and not body composition.

Lagström *et al*. (2020) evaluated the association between HMO composition in maternal milk and child height and weight through to 5 years of age. Milk samples (10 mL) were collected 3 months postpartum by manual expression in the morning from a single breast from 802 Finnish mothers who participated in the Steps to Healthy Development of Children (STEPS) birth cohort study. HMO composition was analyzed by HPLC coupled with fluorescence detection. Child growth data (closest to 3, 6, and 8 months of age and 1, 2, 3, 4, and 5 years of age) were obtained from municipal follow-up clinics, where height and weight are measured according to standardized methods provided by the Finnish Institute for Health and Welfare. Population-specific z-scores for height, weight, and BMI were obtained from growth charts specific to the Finnish population. Associations between HMO concentrations in maternal milk and infant height and weight z-scores were assessed by hierarchical linear mixed models for repeated measurements, adjusted for sex of the infant, mode of delivery, birth weight z-score, maternal pre-pregnancy BMI, and time.

The median concentration of 2'-FL in milk samples collected 3 months postpartum was 2.96 g/L (6,059 nmol/mL). The majority of mothers were characterized as Secretors (87.2%) based on 2'-FL levels in milk samples, which were significantly higher in mothers characterized as Sectors than non-secretors (P<0.001). Significant positive correlations between HMO-bound fucose in Secretor milk and height and weight z-scores in children 3 to 12 months of age and 1 to 5 years of age were reported. Specifically, concentrations of 2'-FL in Secretor milk was positively correlated with height z-scores (coefficient of main effect of 2'-FL of 0.299; 95% CI of 0.042 to 0.416; P=0.016) and weight z-scores (coefficient of main effect of 2'-FL of 0.210; 95% CI of 0.033 to 0.387; P=0.020) in children 3 to 12 months of age, and was nearly significant for height z-scores in children 1 to 5 years of age (coefficient of main effect of 2'-FL of 0.197; 95% CI of -0.002 to 0.397; P=0.053). The study authors noted several limitations, including the collection of milk samples at a single time point (3 months postpartum) even though HMO composition varies throughout lactation, data were unavailable for potential confounding factors (maternal diet and infant morbidity), not all infants were exclusively breastfed, and the median duration of breastfeeding was 10 months. Furthermore, the study authors indicated that the significant association may have been influenced by the study population being primarily composed of Secretors (87.2%) and the near absence of 2'-FL in the milk of non-secretors. The study authors concluded that the composition of HMOs in breast milk may be a mediator of the programming of child growth attributed to breast milk, though evidence of a causal relationship still remains to be investigated.

Relationship Between HMO Composition in Maternal Milk and Infant Growth and Body Composition

The association between HMO composition in maternal milk and infant growth and body composition at 1 and 6 months of age was evaluated by Alderete *et al*. (2015) in 25 mother-infant pairs recruited from a U.S. hospital. Infants were exclusively breastfed for 6 months. Maternal milk was collected following complete expression from a single breast and HMO composition was analyzed by HPLC coupled with fluorescence detection. Infant body composition (percentage fat, total fat, lean mass) and infant growth (crown-to-heel length and weight) were measured at 1 and 6 months. Body composition was measured by dual-energy X-ray absorptiometry, crown-to-heel length was measured in duplicate using a Seca 416 infantometer, and body weight was measured in duplicate using a Seca 728 scale. Associations between HMO concentrations in maternal milk and infant weight and body composition were assessed by multiple linear regression. There was no significant difference in the concentration of 2'-FL in maternal milk collected at 1 month compared to 6 months postpartum. No significant association between the concentration of 2'-FL in maternal milk and infant growth or body composition was reported at 1 or 6 months (significant associations reported for lacto-*N*-fucopentaose, disialyl-lacto-*N*-tetraose, fucosyl-disialyllacto-*N*-hexaose, and lacto-*N*-neotetraose only).

In a more recent exploratory study, Larsson *et al.* (2019) assessed the relationship between HMO composition of breast milk and excessive weight gain in infants exclusively fed breast milk (Larsson *et al.*, 2019). Mother-infant pairs evaluated in the current study were recruited as part of the SKOT III cohort, which included infants 4 to 6 months of age grouped according to their WAZ. Infants in the high weight-gain group (HW group) are defined as having a WAZ >2 and an increment of >+1 SDS in WAZ during the first 5 months postpartum (n=13), whereas infants in the normal weight-gain group (NW group) are defined as having a WAZ between −1.0 andt+ 1.0 SD (n=17). As part of the inclusion criteria, infants had to be exclusively breastfed at least 4 months postpartum. Although the mother-infant pairs were not matched according to gender, birth weight, maternal age, parity, and mode or place of delivery, the study authors reported that the groups were overall well-matched. In addition to anthropometric measurements (weight and height), the body composition of infants was measured at the 5-month visit using a bioelectrical impedance analyzer from which fat-free mass (FFM), fat mass, and fat mass percentage were calculated.

During the same visit, maternal weight and height were measured, whereas maternal pre-pregnancy BMI and gestational weight gain were self-reported. Milk samples were collected using a manual breast pump 5 to 6.5 and 9 months postpartum and consisted of the entire content of both breasts. HMOs were analyzed by HPLC following fluorescent derivatization and mass spectrometry.

The concentration of HMOs in breast milk was first compared between the HW and NW infant groups according to Secretor status of the mother. In the HW group, 8 mothers were Secretors and 3 were non-Secretors, whereas in the NW group, 15 mothers were Secretors and 2 were non-Secretors. Due to the small number of non-Secretors, a separate analysis was not conducted for this group. The concentration of total HMO-bound fucose in milk samples from Secretors was significantly higher in the HW group than the NW group at 5 (P=0.033) and 9 months (P=0.049) postpartum. The concentration of 2'-FL in milk samples from Secretors was higher in the HW group than the NW group, nearing statistical significance at 5 months postpartum (P=0.061), but not 9 months postpartum (P=0.160). However, there was no significant difference in the absolute daily intake of 2'-FL between groups based on 24-hour milk volume at 5 months postpartum[5] (HW group: 3.9 g/day; NW group: 2.4 g/day; P=0.096). Upon combining Secretors with non-Secretors, there was no significant difference in total HMO-bound fucose or 2'-FL between groups. From 0 to 5 months, total HMO, total HMO-bound fucose, and 2'-FL concentrations in milk samples from Secretors had significant positive associations with weight velocity (P=0.016, 0.005, and 0.015, respectively) and fat mass index (P=0.025, 0.018, and 0.024, respectively), and nearly significant positive associations with change in WAZ (P=0.10, 0.08, and 0.09, respectively). Maternal BMI at 5 months had a significant positive association with total HMO, total HMO-bound fucose, and 2'-FL (P=0.033 and 0.017, respectively).

The authors acknowledge that these findings are contradictory to those from randomized controlled trials in infants fed formula supplemented with 2'-FL that included a reference control group of breastfed infants (Marriage *et al.*, 2015; Puccio *et al.*, 2017), where no significant difference in weight gain was observed between groups, and for which weight gain was the primary endpoint. However, they note that the concentration of 2'-FL added to infant formula of up to 1.0 g/L is considerably lower than the average 2'-FL concentration in the NW and HW groups of approximately 3 and 4 g/L in milk from Secretors, respectively. The study authors note that a major limitation of this study is that the relationship between HMO composition and excessive weight gain was evaluated at a single time point only (5 to 6.5 months postpartum), whereas HMO composition of human milk varies during lactation, meaning that HMO composition throughout the period of excessive weight gain was not assessed. The authors speculated that assessment during the period of weight gain could have led to stronger associations; however, it is not possible to conclude on this. Additional limitations were noted by the study authors: infants may have been introduced to complementary foods by 5 months postpartum, the study was not powered for weight gain[6], and statistical analyses did not consider a multiple outcome measures design nor adjust for other milk components. Importantly, there was no significant difference in the daily absolute intake of total HMOs or any of the individual HMO's, including 2'-FL, at 5 months postpartum in breastfed infants based on 24-hour milk volume (total HMO-bound fucose intake not provided) and the sample size in the study (n=13 and 17 per group) was substantially smaller than the sample sizes calculated to investigate weight gain as a primary outcome (n=64 to 66 per group), drawing into question the reliability of the reported statistically significant effects. Therefore, there is no conclusive evidence to suggest a concern for excessive weight gain with

[5] Data not available for 9 months postpartum.

[6] In randomized controlled trials investigating weight gain (g/day) as the primary outcome in infants fed formula supplemented with 2'-FL (Marriage *et al.*, 2015; Puccio *et al.*, 2017), a sample size of 64 to 66 infants in each formula feeding group was calculated to have 80% power to detect a difference in means of ≥3 g/day, assuming a standard deviation of 6 g/day, with a 0.05 2-sided significance level or 0.025 1-sided significance level.

high concentrations of 2'-FL in breast milk (average of 3 to 4 g/L) and no evidence to suggest a safety concern with the intended use level of 2.4 g 2'-FL/L in infant formula.

Binia *et al.* (2021) evaluated the relationship between HMOs and infant growth and adiposity over the first 4 months of lactation in 357 mother-infant pairs from 7 European countries. Infant anthropometry (weight, length, head circumference, and BMI), infant body composition (fat mass and FFM), and HMO composition were assessed at 6 postpartum time points (*i.e.,* birth and 2, 17, 30, 60, 90, and 120 days) in varying numbers of subjects. Maternal milk was collected following complete expression from a single breast and HMO composition was analyzed by liquid chromatography with fluorescence detection (LC-FD). Due to withdrawal from the study for varying reasons, 322 mother-infant pairs were assessed at birth, and 224 mother-infant pairs were assessed at 4 months. Relationships between individual HMO AUC over time (Day 2 to Day 120) and infant anthropometry and body composition parameters at 4 months were assessed using Spearman's rank-order correlations. There were no significant associations between 2'-FL AUC and anthropometric data at 4 months. No significant association between fat mass or fat mass index (FMI) and 2'-FL AUC or any individual HMO concentration over time was found using principal component analysis (PCA). The study authors concluded that "*individual HMO AUC during the first 4 months appears to have no or only moderate effect on infant growth and body composition during this time of exclusive breastfeeding in term-born, healthy growing infants*" (Binia *et al.*, 2021).

The association between HMO intake and infant growth from 0 to 6 months of age in 194 mother-infant breastfeeding pairs (140 exclusively breastfeeding) was investigated by Saben *et al.* (2021). Maternal milk was collected following complete expression from a single breast 2 months after birth and was analyzed by HPLC. Infants' gestational weight gain was calculated between the subject's first study visit and Week 36 of gestation. Infant weight, length, fat mass, and FFM were measured at 2 and 6 months of age. The relationship between HMO intake and infant growth from 2 to 6 months was determined using linear mixed-effects models. No significant associations were reported between 2'-FL consumption and infant growth, length, fat mass, or FFM from 2 to 6 months of age.

Relationship Between Maternal Secretor Status and Diarrhea in Infants

Muthumuni *et al.* 2021 assessed the relationship between maternal and infant Secretor status (*i.e.*, the presence of an active or inactive FUT2 gene) of breastfeeding Caucasian mothers and diarrhea in infants in 4,971 mother-infant pairs. Genetic Secretor status of mothers and infants, breastfeeding status at 6 months (yes/no), and infant diarrhea status at 6 and 18 months (one or more episodes of diarrhea) were determined. Logistic regression was used to determine the relationship between infant FUT2 secretor status and occurrence of infant diarrhea. Infant diarrhea was also assessed in relation to maternal Secretor status. Interaction terms were included in regression models to determine the interactions between maternal Secretor status, infant Secretor status, and breastfeeding. In addition, sensitivity analysis was carried out on 2 or more cases of infant diarrhea from 0 to 6 months or 6 to 18 months of age. It was reported that non-Secretor infants' risk of diarrhea was decreased by 15% [odds ratio (OR): 0.85; 95% confidence interval: 0.72 to 0.99] from 0 to 6 months of age and was decreased by 30% (OR: 0.70; 0.61 to 0.81) from 6 to 18 months of age. There was an association between breastfeeding and reduced risk of diarrhea; 67% (OR: 0.33; 0.24 to 0.44) and 59% (OR: 0.41; 0.35 to 0.49) reduced risk for non-Secretor and Secretor mothers, respectively. The authors concluded that infants being non-Secretors, breastfeeding, and breastfeeding by non-Secretor mothers all reduce the risk of infant diarrhea.

6.5.3 Safety of 2'-FL in Enteral Tube Feeding Formula

The use of 2'-FL as an ingredient in enteral tube feeding formula, intended for patients 11 years of age or older, at use levels of up to 20 g/kg has previously been concluded to be GRAS and notified to the U.S. FDA in GRN 897 (U.S. FDA, 2020b). Data in support of the safety of 2'-FL in formula for enteral tube feeding has previously been summarized in GRN 897 (detailed in response to FDA Question 8 regarding GRN 897), is incorporated by reference, and has been updated and elaborated upon herein.

In GRN 897, the notifier concluded that the safety of 2'-FL for use in enteral tube feeding formula at a use level of 20 g/kg was supported by (i) the low estimated consumption on a body weight basis in comparison to consumption *via* infant formula, (ii) the lack of genotoxicity and systemic toxicity in high-quality published studies of 2'-FL, with reported NOAELs approximately 150 times higher than expected intake from conventional foods and enteral tube feeding formula (*i.e.*, 5.0 to 7.76 g 2'-FL/kg body weight/day in rodents; the highest doses tested) (Phipps *et al*., 2018; van Berlo *et al*., 2018), and (iii) a lack of compound-related adverse effects reported in high-quality clinical studies of 2'-FL (Kajzer *et al*., 2016; Nowak-Wegrzyn *et al*., 2019). In the U.S. FDA's Question 8 to the notifier, it was noted that "*consumers of tube-feeding formulas constitute a vulnerable sub-population, and that the suitability of providing [poorly]-digestible carbohydrate in such formulas may be problematic (e.g., see Tarleton et al.,2013)*" (U.S. FDA, 2020b), and the U.S. FDA requested a narrative supporting the safe use of 2'-FL in enteral tube feeding formula.

In response, the notifier provided additional data in support of the safety of 2'-FL in formula for enteral tube feeding. The notifier reported that Tarleton *et al*. (2013) identified only 2 population groups in which tolerance to poorly-digestible carbohydrates may be reduced, which included patients at high risk for bowel ischemia or severe bowel dysmotility (U.S. FDA, 2020b). The notifier further reported that since these conditions are easily identifiable, the health professional responsible for administration of the enteral tube feeding formula would be aware of the patient's health status and concluded that they concurred with the conclusions of Tarleton *et al*. (2013).

The notifier acknowledged that there are no published studies of the safety or tolerability of 2'-FL as a component of enteral tube feeding formulas, and therefore, considered the results of 17 unique published studies[7] of the safety/tolerability of other poorly-digestible carbohydrates as components of enteral tube feeding formula in a variety of healthy and vulnerable patient populations. An additional study included in the reference list but not in the response to Question 8 of GRN 897 also was identified. These studies are summarized below in Table 6.5.3-1. Briefly, the studies involved administration of partially hydrolyzed guar gum (PHGG), galactomannan, FOS, galactooligosaccharides (GOS), and FOS/GOS mixtures at doses up to 63 g/day. The notifier concluded that the safety of the use of 2'-FL as an ingredient in enteral tube-feeding formula at levels up to 20 g/kg is supported by the lack of test compound-related adverse effects reported in these 17 studies, as well as the Institute of Medicine's conclusion that establishing a tolerable upper intake level for fiber is not necessary due to the unlikelihood of adverse effects due to excessive consumption of fiber (U.S. FDA, 2020b). Upon consideration of the information provided by the notifier, the U.S. FDA responded with no questions regarding the GRAS status of 2'-FL under the conditions of use specified in GRN 897, including use in enteral tube feeding formula at levels up to 20 g/L.

[7] Due to the inclusion of a pair of kin studies (Homann *et al*., 1994, 2004) and a duplication of an additional study (Karakan *et al*., 2007), a total of 17 unique studies of poorly-digestible carbohydrates in enteral feeding formula were included in GRN 897.

Kyowa obtained the original published studies of poorly-digestible carbohydrates in enteral tube feeding formula cited in GRN 897 in order to clarify details of study design and results as presented in GRN 897. In addition, Kyowa conducted a search of the published literature[8] on 27 October 2021 to identify any studies of poorly-digestible carbohydrates in enteral tube feeding formula published since March of 2020. One newly identified study of poorly-digestible carbohydrates in enteral tube feeding formula was identified (Chen *et al.*, 2021) and is included in Table 6.5.3-1 below.

The 19 unique studies of poorly-digestible carbohydrates cited in Table 6.5.3-1 included studies of PHGG, galactomannan, FOS and/or GOS, or polydextrose in healthy adults or children and adults, children, and infants with a range of chronic or acute medical conditions. In these studies, no test product-related adverse effects were reported with respect to the measured parameters, including those related to clinical outcomes, immune function, fecal characteristics (*e.g.*, frequency or consistency), or standard safety parameters (*i.e.*, hematology, clinical chemistry, or vital signs). Some mild, transient symptoms of gastrointestinal intolerance considered typical and expected following supplementation with soluble fiber (*i.e.*, flatulence, abdominal distension, abdominal pain, diarrhea) were reported in several studies. The reported gastrointestinal symptoms occurred upon supplementation with up to 24 g PHGG/day in post-operative or critically ill adults, consumption of 30 g scFOS/day by healthy adults, or administration of approximately 1.22 g FOS/day to children (1 to 12 years of age) undergoing chemotherapy for stage 1 to 3 cancer (Homann *et al.*, 1994, 2004; Fussell *et al.*, 1996; Garleb *et al.*, 1996; Zheng *et al.*, 2006). In all 4 of these studies, the authors concluded that overall, the intervention products were well tolerated and were considered to be beneficial with respect to clinical outcomes (Homann *et al.*, 1994, 2004; Fussell *et al.*, 1996; Garleb *et al.*, 1996; Zheng *et al.*, 2006).

Kyowa agrees with the conclusions presented in GRN 897, *i.e.,* thatthe results of the identified studies of poorly-digestible carbohydrates at doses up to 63 g/day in enteral tube feeding formula support the safety of 2'-FL for use in enteral tube feeding formula at the intended use level of 20 g/kg. Based on the totality of evidence pertaining to the safety of 2'-FL and the use of poorly-digestible carbohydrates in enteral tube feeding formula, Kyowa concludes that the safety of their 2'-FL ingredient in formula for enteral tube feeding at a use level of 20 g/L (resulting in a total intake of 10 g/day) is supported by the safety profile of 2'-FL and the safety of poorly-digestible carbohydrates in general as ingredients in enteral tube feeding formula at levels that exceed the recommended intake of Kyowa's 2'-FL from the intended use in enteral tube feeding formula.

[8] The databases searched include: AdisInsight: Trials, AGRICOLA, AGRIS, Allied & Complementary Medicine™, BIOSIS® Toxicology, BIOSIS Previews®, CAB ABSTRACTS, Embase®, Foodline®: SCIENCE, FSTA®, MEDLINE®, NTIS: National Technical Information Service, ToxFile®, Toxicology Abstracts, and Toxicology Abstracts. Terms pertaining to human exposure *via* tube-feeding formula were used with terms intended to capture poorly-digestible carbohydrates (*i.e.,* names, synonyms, abbreviations, and CAS numbers) included in the notifier's response to FDA's Question 8 on Page 39 GRN 897.

Table 6.5.3-1 Studies from GRN 897

Patient Population and Study Design	Dose or Concentration and Study Duration	Results Relevant to Safety	Reference
Studies of PHGG			
11 healthy men P, R, DB, PC	ETFF providing 0 or 15 g PHGG/day 18 days	• No compound-related adverse effects on fecal wet and dry weights, fecal moisture content, fecal pH, and stool frequency. • No adverse events reported. Authors concluded *"despite significant differences in mean transit time, few differences in other parameters of bowel function were observed when healthy subjects consumed enteral formula diets containing 0 g of fiber and 15 g of total dietary fiber as modified guar and soy".*	Lampe *et al.* (1992)
12 healthy men (mean age = 29 years) R, DB, CO	Liquid formula diet providing 0 or 42 g PHGG/day 7 days	• Significantly increased colonic transit time (*vs.* washout period or formula without fiber; not considered to be an adverse effect). • No effect on stool consistency or frequency reported.	Meier *et al.* (1993)
10 healthy adults R, DB, CO	ETFF with 42 to 63 g PHGG/day 7 days	• No reports of intolerance. • No adverse effects on hemoglobin, hematocrit, total and differential white blood cell count, Na, K, Mg, Cl, ALT, AST, GGT, alkaline phosphatase, bilirubin, or creatinine.	Alam (1993)
100 postoperative subjects (mean age = 59 to 70 years); 30 administered TEN and 70 administered enteral supplementation P, R, DB, PC	TEN: standard ETFF or ETFF with 24 g PHGG/day Enteral supplementation: standard ETF or ETF with 20 g PHGG/day ≥5 days	• Increased but well-tolerated flatulence (both PHGG groups), without bloating or cramping. • Total number of adverse gastrointestinal effects not significantly different between PHGG and standard ETF groups. The authors reported that *"The total number of GI-side effects was not different in the two groups (17 in each group)".*	Homann *et al.* (1994, 2004)
57 critically ill adults (with recent abdominal surgery/ trauma, cerebral trauma, head/neck surgery, multiple fractures, or vascular surgery) P, R, DB, PC	ETFF with 0 or 14 g PHGG/L formula 5 to 14 days	• No adverse effects with respect to diarrhea, albumin, transthyretin, or flatulence. • Abdominal distension observed significantly more often in PHGG group, although authors noted that this was not clinically significant. • PHGG was generally well tolerated.	Fussell *et al.* (1996)
12 subjects (age NR) with type 1 diabetes PC, CO	480 mL ETFF consumed over 4 hours (dose or concentration of PHGG NR)	• Monitoring or reporting of adverse effects or adverse events not reported.	Peters and Davidson (1996)
25 ICU patients (mean age = 68.5 ± 13.1 years) with severe sepsis and septic shock P, R, DB, PC	Standard ETFF or ETFF with 22 g PHGG/L (daily dose NR) 6 to 21 days	• No adverse effects with respect to diarrhea, sepsis-related mortality, or duration of ICU stay. Authors concluded *"Fiber treatment was well-tolerated and did not affect glucose control".*	Spapen *et al.* (2001)

Table 6.5.3-1 Studies from GRN 897

Patient Population and Study Design	Dose or Concentration and Study Duration	Results Relevant to Safety	Reference
20 adult ICU patients (with ≥3 liquid stools/day and a variety of health conditions/injuries) P, R, DB, C	ETFF With 22 to 39 g PHGG/day (22 g PHGG/L) 4 days	• No compound-related adverse effects on number of liquid stools, tolerance, or incidence or severity of gastrointestinal symptoms (including flatulence, vomiting, constipation. • Significant decrease from baseline in number of liquid stools.	Rushdi *et al.* (2004)
Studies of Galactomannan			
20 elderly subjects (bed-ridden) Open-label	ETFF with 7 g galactomannan/day (1st week); dose increased by 7 g/day each week until 4th week (28 g/day) 4 weeks	• No compound-related adverse effects on serum diamine oxidase activity, fecal water content, frequency of normal stools, frequency of bowel movements, number of aerobic bacteria, fecal pH, fecal SCFA, total bacteria or anaerobe counts, body weight, total serum protein, prealbumin, transferrin, retinol-binding protein, total cholesterol, triacylglycerol, iron, copper, or zinc. • No adverse events reported. Authors concluded that soluble dietary fiber is *"useful for controlling spontaneous, favorable bowel movement"*.	Nakao *et al.* (2002)
Studies of FOS			
30 patients (mean age = 46.1 ± 14.0 years) with severe acute pancreatitis R,dDB,dPC	ETFF with 24 g fiber (containing approximately 50% scFOS)/day 2 days	• No compound-related adverse effects on duration of enteral feeding or hospital stay, pancreatitis severity scores, mortality, or overall complications. • Formula was well tolerated with no reported adverse effects or adverse events.	Karakan *et al.* (2007)
14 children (1 to 15 years of age) with compromised gut function receiving 75 to 100% of calories *via* ETF R,dDB,dCO	ETFF with 3.5 g FOS/day (3.5 g FOS/L) 14 days	• No compound-related adverse effects with respect to stool quality, vomiting, abdominal pain, or weight gain. Authors concluded *"This study showed that a peptide-based formula containing fiber was as well-tolerated as a fiber-free formula in a small population of children with gastrointestinal impairments"*.	Khoshoo *et al.* (2010)
27 healthy college students R, DB,dC	ETFF with 0, 15, or 30 g scFOS/day (0, 5, or 10 g/L formula) 14 days	• No compound-related adverse effects on body weight, clinical chemistry, fecal short-chain fatty acids, fecal pH, fecal dry matter, reported adverse effects (nausea, cramping, distension, vomiting, diarrhea, and regurgitation). • Increased flatulence in 30 g/day group (during first 4 days of intervention). • One withdrawal from high-dose group due to unspecified intolerance. • scFOS-containing formulas were well-tolerated. Authors concluded that *"these results indicate that [scFOS] does not compromise serum chemistry profiles, is well tolerated particularly at an intake of 15 g/d and would serve as a bifidogenic factor when incorporated into a liquid enteral product"*.	Garleb *et al.* (1996)

Table 6.5.3-1 Studies from GRN 897

Patient Population and Study Design	Dose or Concentration and Study Duration	Results Relevant to Safety	Reference
94 critically ill children (1 to 3 years of age) on mechanical ventilation R, DB, PC	Control ETFF or ETFF with 2.6 g oligofructose/inulin and 2.8 g acacia gum/L, DHA, and 5 strains of live microorganisms ≤14 days	• No compound-related adverse effects on caloric intake, abdominal distension, vomiting, stool frequency, or fecal microbiota. Authors concluded that the study product is safe and well tolerated by children in ICU.	Simakachorn *et al.* (2011)
67 children (1 to 12 years of age) with stage 1 to 3 cancer and undergoing chemotherapy P, R, DB, PC	Standard ETFF or ETFF with FOS (2 g/L; 1.22 ± 0.24 g/day; 60 ± 20 mg/kg bw/day) 13 to 30 days	• No compound-related adverse effects on fecal microbiota, biomarkers of immunologic status (*i.e.,* cytokines and cell counts), nutritional status, weight, blood pressure, heart rate, body temperature, respiratory rate, prognostic inflammatory and nutritional index, stool characteristics, and standard hematological and biochemical parameters. • Transient gastrointestinal effects: rectal discomfort (1/32 in FOS group), mild flatulence (3/32 in FOS group; 2 reported in association with abdominal pain), mild diarrhea (1/32 in FOS group), nausea (12/35 in control group and 11/32 in FOS group). • One adverse event: 1 subject in FOS group had diarrhea and complained of abdominal pain on study Day 3 and was withdrawn from the study (subject had been non-compliant with study protocol from Days 1 to 3). • Authors noted a lack of gastrointestinal discomfort and concluded that "*Both enteral formulas were well tolerated and accepted*".	Zheng *et al.* (2006)
Studies of GOS or GOS/FOS Mixtures			
154 preterm infants (gestational age <33 weeks) P, R, DB, PC, MC	Standard formula or formula with 8 g scGOS:lcFOS (9:1)/L ~8 weeks or until hospital discharge	• No compound-related adverse effects on tolerance; gains in weight, length, or head circumference; stool frequency or characteristics; fecal microbiota; gastrointestinal signs; or overall water balance (based on concentrations of serum sodium and creatinine). Authors concluded that "*Prebiotic supplementation appears safe and may benefit enteral tolerance in the most immature infants*".	Modi *et al.* (2010)
23 elderly subjects (bedridden with a variety of chronic health conditions) P, R, DB, PC	Standard ETFF or ETFF with fermented milk, GOS (4 g/day), and prebiotic bifidogenic growth stimulator (0.4 g/day) 10 weeks	• No compound-related adverse effects with respect to hematology, clinical chemistry, fecal microbiota, antibody response to influenza vaccine, or plasma cytokine levels. • No adverse events reported.	Akatsu *et al.* (2016)

Table 6.5.3-1 Studies from GRN 897

Patient Population and Study Design	Dose or Concentration and Study Duration	Results Relevant to Safety	Reference
50 preterm neonates with hyperbilirubinemia P, R, DB, PC	Standard ETFF or ETFF with 9:1 ratio of scGOS:icFOS (initially 0.5, increased to 1.5 g/kg bw/day) 1 week	• No adverse effects with respect to bilirubinemia or stool frequency. Authors concluded "*Prebiotic oligosaccharides increase stool frequency, improve feeding tolerance and reduce bilirubin level in preterm neonates and therefore can be efficacious for the management of neonatal hyperbilirubinemia*".	Armanian *et al.* (2016)
113 infants with gestational age <32 weeks or birth weight <1,500 g P, R, DB, PC	Breast milk or formula alone or with scGOS, lcFOS, and pectin-derived acidic oligosaccharides (dose or concentration NR) 28 days	• No adverse effects on response to influenza vaccination. • Monitoring or reporting of adverse events not reported.	van den Berg *et al.* (2015)
Studies Identified in Literature Search Conducted 27 October 2021			
51 adults with severe acute pancreatitis P, R, single-blind, C	Standard ETFF or ETFF with 20 g polydextrose/day	• No compound-related adverse effects on feeding intolerance, symptoms and signs of gastrointestinal tolerance (abdominal distension, vomiting, diarrhea, constipation, gastrointestinal bleeding, bowel sounds, intra-abdominal pressure), other signs of gastrointestinal health (flatulence, bowel habit, intestinal barrier function, gastrointestinal hormones), or clinical outcomes. • No adverse events reported. Authors conclusion: soluble dietary fiber is well tolerated and improves clinical outcomes in patients with severe acute pancreatitis.	Chen *et al.* (2021)

ALT= alanine aminotransferase; AST= aspartate aminotransferase; C = controlled; CO= crossover; DB= double-blind; DHA = docosahexanoic acid; ETFF = enteral tube feeding formula; FOS = fructooligosaccharides; GGT = *gamma*-glutamyltransferase; GOS = galactooligosaccharides; GRN = GRAS Notice; IBS = irritable bowel syndrome; ICU= intensive care unit; lc= long-chain; MC= multi-center; NR = not reported; P = prospective; PC= placebo-controlled; PHGG = partially hydrolyzed guar gum; R = randomized; sc = short-chain; SCFA = short-chain fatty acids; TEN = total enteral nutrition.

6.6 Other Considerations – Use of 2'-FL in Combination with Other HMOs or Poorly-Digestible Carbohydrates

Kyowa is not a manufacturer of infant formula; however, the company considers it likely that infant formula manufacturers may use a combination of HMOs, or other poorly digestible carbohydrates, to produce infant formula products that are more compositionally similar to human milk. Kyowa acknowledges that symptoms of gastrointestinal intolerance have been reported upon consumption of large amounts of poorly-digestible carbohydrates, especially in sensitive populations, including infants. Kyowa anticipates that their HMO ingredients, including 2'-FL, may be used as ingredients in infant formula in combination with other HMOs in order to provide a variety of HMOs at concentrations that are within the natural variation of concentrations found in human milk. The safety and expected tolerability of the combined intake of different HMOs and other poorly-digestible carbohydrates have been addressed in previous GRAS notices to which the U.S. FDA responded with no questions (GRNs 815, 833, 880, 881, 932, and 951).

As noted above in Section 1.3, the proposed use level of Kyowa's 2'-FL in infant formula was chosen by Kyowa to align with the mean concentration of 2'-FL in human milk (*i.e.,* 2.4 g 2'-FL/L). This use level is well within the range of concentrations in human milk, with corresponding intakes from all intended uses within the ranges of infant consumption of 2'-FL *via* human milk. Other HMOs also are GRAS for use in infant formula (with no questions from the U.S. FDA), including 3'-SL, 6'-SL, 3'-FL, 2'-FL/DFL, LNT, and LNnT, at use levels intended to be reflective of the mean concentrations of the individual HMOs in human milk, taking into account natural variation among women with differing Lewis/Secretor genotypes and at various lactational stages (GRNs 659, 815, 833, 880, 881, 932, and 951). Since each individual HMO would be used in infant formula at a level comparable to its mean concentrations in human milk, the combined intake of these HMOs as ingredients in infant formula would be expected to be similar to their intake *via* human milk. The combined intake of the HMOs that are GRAS for use in infant formula would therefore be expected to be safe and well tolerated based on their history of consumption *via* human milk.

Kyowa notes that the results of 4 intervention studies in healthy, singleton, term infants provide support for the safety and tolerability of consumption of combinations of HMOs that are individually present at use levels that are within their natural variation in human milk or present with other poorly digestible carbohydrates (see Section 6.5.1 above). In these studies, no between-group differences considered to be adverse or clinically relevant were reported among infants consuming formula supplemented with up to 1.0 g 2'-FL/L with 2 g scFOS/L, up to 1.4 g GOS/L, or 0.5 g LNnT/L for up to 6 months (Marriage *et al*., 2015; Goehring *et al*., 2016; Kajzer *et al*., 2016; Puccio *et al*., 2017). The study authors concluded that the study products were safe and well-tolerated, with no safety concerns noted.

The use of Kyowa's 2'-FL in infant formula is intended to be substitutional to other 2'-FL ingredients produced by other manufacturers and currently on the U.S. market; therefore, additive consumption of 2'-FL, beyond the estimated consumption levels detailed in Section 3.4 above, is not expected.

Kyowa cannot provide input on the levels of other poorly-digestible carbohydrates that may be used in infant formula in combination with their HMO ingredients, as Kyowa is not a manufacturer of infant formula. However, any new infant formula containing new HMOs, a new HMO combination, or a new combination of HMOs and other poorly-digestible carbohydrates in the U.S. would be subject to Section 412 of the Federal Food, Drug, and Cosmetic Act (FFDCA) (21 USC §350(a)). According to Section 412(d)(1) of the FFDCA, a manufacturer must notify the U.S. FDA ≥90 days before marketing a new infant formula; this notice must include descriptions of any reformulation or change in processing of the infant formula. The manufacturer would therefore need to provide the U.S. FDA with information supporting that the combination of poorly-digestible carbohydrates, including HMOs, intended to be used in the infant formula would be well-tolerated. Therefore, Section 412 of the FFDCA would ensure that any combination of HMOs or other poorly-digestible carbohydrates would be supported by tolerance and safety testing in infants.

6.7 Allergenicity

The allergenic potential of Kyowa's 2'-FL is expected to be very low. This lack of allergenic potential is supported by analytical data demonstrating that, similar to 2'-FL ingredients notified to the U.S. FDA, Kyowa's final 2'-FL product does not contain the production strain or residual proteins, both of which are removed during the purification steps of the manufacturing process (*via* microfiltration and ultra-filtration). The absence of the production organism in Kyowa's final 2'-FL ingredient was demonstrated using PCR (see Section 2.3.3.3.1). Kyowa's final 2'-FL ingredient produced with a genetically modified strain of *E. coli* W is specified to contain ≤10 mg/kg residual protein, and the batch analysis of 6 lots of Kyowa's 2'-FL ingredient yielded residual protein levels equal to or below the limit of detection of 1 mg/kg (0.0001%; see Table 2.3.3.1-1).

Kyowa has conducted 2 tests of their final 2'-FL ingredient (Lot A) to assess the presence of milk proteins. These tests were conducted using 2 enzyme-linked immunosorbent assay (ELISA) test kits [FASPEK ELISA II Milk (Casein; Morinaga Institute of Biological Science, Inc.) and FASTKIT ELISA Ver. III MILK (NH Foods Ltd.)], both of which have quantification limits of 1.0 µg/g. Milk proteins were not detected with either ELISA test kit, demonstrating that milk proteins are effectively removed during the purification process and are not present in Kyowa's final 2'-FL ingredient.

No published reports of sensitization, case reports of allergic reactions, or allergenicity studies on 2'-FL were identified in a comprehensive and detailed search of the published scientific literature that was conducted through 07 December 2021 to identify studies relevant to the safety of 2'-FL.

Kyowa's 2'-FL manufactured with a genetically modified strain of *E. coli* W was concluded to be of low allergenic risk.

6.8 Basis for GRAS

The conclusion that 2'-FL produced by fermentation using a genetically modified strain of *E. coli* W is GRAS for use as an ingredient in non-exempt infant formula, conventional foods, and foods for special dietary uses is on the basis of scientific procedures.

Kyowa's 2'-FL has been demonstrated by LC-MS, ^{1}H NMR, and ^{13}C NMR to be structurally and chemically identical to 2'-FL that is naturally present in human breast milk. On the basis of the chemical and structural identity to 2'-FL from human milk, the intakes of Kyowa's 2'-FL under the conditions of intended use in comparison to the natural background dietary exposure to 2'-FL from the consumption of human milk is pivotal in the assessment of the safety of Kyowa's 2'-FL ingredient. Natural background dietary intakes of 2'-FL in infants from the consumption of human milk are higher than those estimated under the proposed conditions of use of Kyowa's 2'-FL and support the safety of Kyowa's 2'-FL ingredient under the proposed conditions of use. As 2'-FL intakes from all proposed conditions of use are within background exposure to 2'-FL from human milk in infants, a vulnerable population group, 2'-FL is considered to be safe for all population groups.

The safety of Kyowa's 2'-FL ingredient is supported by the results of published safety studies of 2'-FL previously evaluated by various experts qualified by scientific training and experience to evaluate the safety of food ingredients (GRN 546, 571, 650, 735, 749, 815, 852, 897, and 929), as well as by the U.S. FDA (U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a,b, 2020d, 2021) and EFSA (EFSA, 2015). Additional safety studies published subsequent to the latest GRAS notice for 2'-FL submitted to the U.S. FDA also were considered supportive of safety.

The results of unpublished toxicology studies of Kyowa's 2'-FL ingredient were considered as corroborative evidence of the safety of Kyowa's 2'-FL ingredient.

6.9 GRAS Panel Evaluation

Based on the above data and information presented herein, Kyowa Hakko Bio Co., Ltd. has concluded that the intended uses of 2'-FL as an ingredient in non-exempt infant formula, conventional foods, and foods for special dietary uses, as described in Section 1.3 are GRAS, on the basis of scientific procedures.

This GRAS conclusion 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 GRAS Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The GRAS Panel consisted of the following qualified scientific experts: Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Robert J. Nicolosi, Ph.D. (University of Massachusetts Lowell; R.J. Nicolosi, LLC), and Steven L. Taylor, Ph.D. (University of Nebraska-Lincoln; Taylor Consulting LLC).

The GRAS Panel, convened by Kyowa, independently and critically evaluated all data and information presented herein, and also concluded that 2'-FL is GRAS for use as an ingredient in non-exempt infant formula, conventional foods, and foods for special dietary uses as described in Section 1.3, based on scientific procedures. A summary of data and information reviewed by the GRAS Panel, and evaluation of such data as it pertains to the proposed GRAS uses of 2'-FL is presented in Appendix A.

6.10 Conclusion

Based on the above data and information presented herein, Kyowa Hakko Bio Co., Ltd. has concluded that 2'-Fucosyllactose (2'-FL) is GRAS, on the basis of scientific procedures, for use as an ingredient in non-exempt infant formula, conventional foods, and foods for special dietary uses as described in Section 1.3. General recognition of Kyowa's GRAS conclusion 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 food, who similarly concluded that the proposed uses of 2'-FL are GRAS on the basis of scientific procedures.

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*.

Part 7. §170.255 List of Supporting Data and Information

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APPENDIX A
GRAS Panel Consensus Statement

GRAS Panel Consensus Statement Concerning the Generally Recognized as Safe (GRAS) Status of 2'-Fucosyllactose for Use in Infant Formula, Conventional Foods, and Foods for Special Dietary Uses

24 June 2021

INTRODUCTION

Kyowa Hakko Bio Co., Ltd. (Kyowa) intends to market 2'-fucosyllactose (2'-FL), produced by microbial fermentation using a genetically modified strain of *Escherichia coli* W, as an ingredient for addition to infant formula, specified conventional food products, and foods for special dietary uses in the United States (U.S.). Kyowa convened a panel of independent scientists (GRAS Panel), qualified by their relevant scientific training and experience in the safety evaluation of food ingredients, to conduct a critical and comprehensive evaluation of the available pertinent data and information on 2'-FL, and to determine whether the proposed uses of Kyowa's 2'-FL would be Generally Recognized as Safe (GRAS) based on scientific procedures. For the purposes of the GRAS Panel's evaluation, "safe" or "safety" indicates that there is a reasonable certainty of no harm under the intended conditions of use of the ingredient in foods, as stated in 21 CFR §170.3(i) (U.S. FDA, 2020a). The GRAS Panel consisted of the below-signed qualified scientific experts: Joseph F. Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine), Robert J. Nicolosi, Ph.D. (University of Massachusetts Lowell; R.J. Nicolosi, LLC), and Steven L. Taylor, Ph.D. (University of Nebraska-Lincoln; Taylor Consulting LLC).

The GRAS Panel was selected and convened in accordance with the U.S. Food and Drug Administration's (FDA's) *Draft Guidance for Industry: Best Practices for Convening a GRAS Panel* (U.S. FDA, 2017). Kyowa confirms that prior to convening the GRAS Panel, all reasonable efforts were made to identify and select a balanced GRAS Panel with expertise in appropriate scientific disciplines deemed necessary for the safety evaluation of 2'-FL, and efforts were placed on identifying conflicts of interest or relevant appearance issues that would potentially bias the outcome of the deliberations of the GRAS Panel; no such conflicts of interest or appearance of conflicts were identified. The GRAS Panel members received reasonable honoraria as compensation for their time, and honoraria provided to the GRAS Panel were not contingent upon the outcome of the GRAS Panel's deliberations.

The GRAS Panel, independently and collectively, critically examined a comprehensive package of publicly available scientific information and data, both favorable and unfavorable, relevant to the safety evaluation of Kyowa's 2'-FL under the intended conditions of use that was presented to the GRAS Panel in a dossier titled "*Documentation Supporting the Evaluation of 2'-Fucosyllactose as Generally Recognized as Safe (GRAS) for Use in Food*" (dated 24 June 2021). Publicly available scientific information and data included published and unpublished safety studies referenced in GRAS Notices of 2'-FL previously evaluated by qualified scientific experts including the U.S. FDA (GRN 546, 571, 650, 735, 749, 815, 852, 897, and 929), and any new scientific literature that has since been published through to April 2021 (U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a,b, 2020b, 2021). The GRAS Panel also reviewed unpublished studies of 2'-FL sponsored by Kyowa. Information and data evaluated by the GRAS Panel included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, product specifications, supporting analytical

data, the intended conditions of use, the estimated exposure under the intended conditions of use, the history of safe consumption from human breast milk, and the safety of 2'-FL.

Following independent and collective critical evaluation of such data and information, the GRAS Panel unanimously concluded that under the conditions of intended use described herein, 2'-FL produced by microbial fermentation using a modified strain of *E. coli* W, meeting appropriate food-grade specifications, and manufactured in accordance with current Good Manufacturing Practice (cGMP), is GRAS based on scientific procedures. A summary of the basis for the GRAS Panel's conclusion is provided below.

IDENTITY, PRODUCTION ORGANISM, MANUFACTURING, SPECIFICATIONS, AND BATCH ANALYSES

2'-FL is a naturally occurring oligosaccharide in human milk, synthesized from lactose in the mammary gland (Castanys-Muñoz *et al.*, 2013). It is the second most abundant glycan in human milk following lactose, and the most abundant human milk oligosaccharide (HMO) (Castanys-Muñoz *et al.*, 2013). The identity of Kyowa's 2'-FL ingredient was evaluated by high-performance liquid chromatography-mass spectrometry (HPLC-MS), proton nuclear magnetic resonance spectroscopy (^{1}H NMR), and carbon-13 nuclear magnetic resonance spectroscopy (^{13}C NMR). The GRAS Panel critically evaluated chromatograms and spectra demonstrating that Kyowa's 2'-FL (Lot A) is chemically and structurally identical to 2'-FL from human breast milk (Agilent Technologies, Lot No. A522602).

Kyowa's 2'-FL ingredient is produced by microbial fermentation from a genetically modified strain of *E. coli* W. The GRAS Panel reviewed data pertaining to the safety of the host organism and critically evaluated the genetic modifications applied to *E. coli* W for the biosynthesis of 2'-FL. The host organism, *E. coli* W, has been deposited in the American Type Culture Collection (ATCC 9637 – ATCC, 2021a), and is 1 of 4 *E. coli* strains designated safe for laboratory use (Archer *et al.*, 2011). These safe strains are designated as Risk Group 1 organisms according to biological safety guidelines (Archer *et al.*, 2011; ATCC, 2021a), as they are well-characterized and do not cause disease in healthy adult humans (NIH, 2019), and do not colonize the human gut (Bauer *et al.*, 2008). *E. coli* W is non-toxigenic and non-pathogenic, as it lacks genes encoding toxins and genes encoding pathogenic determinants have been mutationally inactivated or are missing key components required for pathogenicity (Archer *et al.*, 2011).

The production strain has been optimized for the production of 2'-FL from the starting materials, lactose and glucose. Host modifications were achieved using a modified lambda red recombinase system (Datsenko and Wanner, 2000), a common technique used to make targeted genetic modifications (including insertions and deletions) in *E. coli* at loci specified by flanking homology regions (Murphy, 1998; Yu *et al.*, 2000; Sharan *et al.*, 2009). The production strain contains 1 heterologous gene sequence (encoding α-1,2 fucosyltransferase) originating from a defined donor organism that is inserted into the chromosomal DNA of the host organism. The gene encoding α-1,2 fucosyltransferase originates from *Helicobacter mustelae* (ATCC 43772 ATCC, 2021b), which is a gastric pathogen of ferrets (Fox *et al.*, 1990). Although the gene encoding α-1,2 fucosyltransferase is cloned from chromosomal DNA from a pathogenic strain, no unspecified DNA is expected to be associated with the transfer of the gene encoding α-1,2 fucosyltransferase as the DNA insert has been mutated (while still maintaining the intended enzyme activity), is well-characterized, and is confirmed to consist of the desired sequence only. Furthermore, the expression product α-1,2 fucosyltransferase has a well-defined function in the biosynthesis of 2'-FL and is not associated with any potential toxicity or pathogenic traits of the donor organism. Host modifications also include the deletion of 5 gene sequences which serve as insertion loci for the inserted gene described above. The final production strain is selected using levansucrase as a counter-selectable marker, an enzyme

that catalyzes the hydrolysis of sucrose, preventing the growth of the production organisms in the presence of sucrose (Pelcic *et al.*, 1996).

The GRAS Panel critically reviewed details of the manufacturing process of 2'-FL, which involves 2 main steps: fermentation and purification. Kyowa has stated that the manufacturing process is controlled by a Hazard Analysis and Critical Control Point (HACCP) plan and in accordance with cGMP as established by 21 CFR §117 (U.S. FDA, 2020a). The fermentation components used in the manufacture of 2'-FL are food-grade and considered safe and suitable for their intended uses in food and/or were previously determined to be GRAS for their intended use and are used consistent with cGMP requirements. The fermentation media used for culturing the genetically modified strain of *E. coli* W contains nutrient sources and ingredients that are commonly used in microbial growth media. Kyowa also confirmed that all raw materials and processing aids are of food-grade quality and are used in accordance with an applicable federal regulation or have been concluded to be GRAS for their respective use. The fermentation process is conducted in chemically defined nutrient media under sterile and controlled conditions (*e.g.*, time, temperature, pH, and feeding rate). The production strain obtained from a frozen cell bank is initially cultured in flask seed culture medium followed by a factory seed culture medium. After reaching a specific optical density, the main fermentation medium is first inoculated with the production strain and fermented in the presence of glucose. Following depletion of glucose, glucose and lactose are added to the fermentation media for the production of 2'-FL, which is secreted into the culture medium. The production of 2'-FL is stopped by sterilization, after which the broth is cooled and acidified.

During the purification processes, the intact production strain cells are removed *via* microfiltration. The obtained solution is then passed through a series of cationic resin and anionic resin ion exchangers to remove cations, anions, minerals, and organic impurities. The concentrated solution is decolorized with activated carbon, and then filtered in a series of filtration steps using microfiltration membranes and an ultra-filtration membrane to remove endotoxins, as well as any residual protein, organic impurities, and production organism not removed by the cationic/anionic exchange resins. The obtained solution is then further concentrated, spray-dried, homogenized using an air blender, and passed through a sieve to remove foreign materials to obtain the final 2'-FL ingredient.

Kyowa has established food-grade physical, chemical, heavy metal, and microbiological specifications for their 2'-FL ingredient (see Table A-1 of Attachment A). Specification limits for 2'-FL purity and related carbohydrate impurities are similar to those established for other 2'-FL ingredients that have been concluded to be GRAS, demonstrating that Kyowa's 2'-FL is compositionally similar to other 2'-FL ingredients permitted on the U.S. market. Specifically, the GRAS Panel noted Kyowa's 2'-FL ingredient contains ≥82% 2'-FL (on a dry basis), contains small amounts of other carbohydrates (≤1 to 5% for each specified carbohydrate on a dry weight basis), and low levels of water (≤9.0 w/w%) and residual protein (≤10 mg/kg). Most specification parameters are evaluated using nationally or internationally accepted validated methods (United States or Japanese Pharmacopoeia; International Organization for Standardization).
Kyowa confirmed that internal methods, including the identification and quantification of 2'-FL and other carbohydrates and quantification of residual protein, were concluded to be suitable. The GRAS Panel critically evaluated analytical results and representative impurity profiles of 6 lots of 2'-FL (5 of which were non-consecutive), which demonstrate that the manufacturing process results in a consistent product that meets specifications.

Kyowa's final 2'-FL ingredient also was assessed for residual production organism and residual production organism-derived DNA in accordance with the European Food Safety Authority's (EFSA's) *Guidance on the characterization of microorganisms used as feed additives or as production organisms* (EFSA, 2018). The results of these analyses on 3 lots of the 2'-FL ingredient demonstrate that the production organism is absent from the final 2'-FL ingredient, and that there is no detectable residual DNA (limit of quantification of 4 μg/kg or 4 ppb) in the final 2'-FL ingredient.

The GRAS Panel critically reviewed bulk stability data of 2'-FL under accelerated conditions (40 ± 2°C; 75 ± 5% relative humidity) and real-time conditions (25 ± 2°C; 60 ± 5% relative humidity). Both studies are ongoing, with results available for 2'-FL stored for up to 6 months under accelerated conditions and for up to 12 months under standard conditions. In both studies, 2'-FL was stored in polyethylene bags within an aluminum foil bag, which are similar packaging materials intended to be used for the storage and distribution of the commercial product. Parameters evaluated included appearance, purity, water, water activity, pH, and levels of other carbohydrates. Available data demonstrate 2'-FL is stable, with no measurable loss. Additionally, the water activity of 2'-FL was considerably lower than 0.88 at all time-points of evaluation and conditions of storage, indicating that microbial growth or toxin formation in Kyowa's 2'-FL ingredient is unlikely. The results of the accelerated stability study support a shelf-life of 3 years. The GRAS Panel notes that Kyowa's 2'-FL ingredient is compositionally similar to other 2'-FL ingredients that have been concluded to be GRAS, and the stability profile of Kyowa's 2'-FL ingredient when incorporated into food matrices is expected to be the same as other 2'-FL ingredients currently included on the GRAS inventory list.

HISTORY OF SAFE CONSUMPTION, INTENDED USE, AND ESTIMATED EXPOSURE

The GRAS Panel noted that 2'-FL has an established history of safe consumption by breastfed infants, as it is the most abundant HMO in human milk (Castanys-Muñoz *et al.*, 2013). Several factors influence the levels of 2'-FL in human milk, although genetic traits, namely Secretor status and Lewis blood groups, are the main sources of variation (Kumazaki and Yoshida, 1984; Thurl *et al.*, 1997, 2010; Stahl *et al.*, 2001). The Secretor gene *FUT2* encodes α(1,2)-fucosyltransferase, which catalyzes the biosynthesis of 2'-FL, whereas the Lewis gene *FUT3* encodes fructosyltransferases that catalyze α1-3 or α1-4 rather than α1-2 linkages (reviewed Castanys-Muñoz *et al.*, 2013). Both genes have a functional dominant allele and a non-functional recessive allele, resulting in 4 distinct genetic groups. Specifically, 2'-FL levels in human milk are highest in Secretors *versus* non-Secretors, as well as Secretors who are Lewis-negative *versus* Secretors who are Lewis-positive. The levels of 2'-FL in human milk have previously been comprehensively reviewed and summarized in Section IV.B.1 of GRN 546 (Glycom A/S, 2014; U.S. FDA, 2015a). The average concentration of 2' FL from pooled mature human milk samples collected from Lactation Days 5 to 100 (considering different demographic groups and phenotypes) was determined to range between 1.10 and 4.26 g/L, with a mean concentration of 2.4 g/L. The maximum concentration of 2'-FL in mature milk was determined to be 7.0 g/L in Secretor milk.

Exposure to 2'-FL on a body weight basis was calculated based on maternal milk levels described above, assuming a standard infant body weight of 6.7 kg (WHO Growth Chart[1]; average of 50th percentile for boys and girls at 4 months) and a milk consumption of 1.2 L/day (Butte *et al.*, 2002; da Costa *et al.*, 2010; Nielsen *et al.*, 2011; EFSA, 2013). The resulting intake of 2'-FL from mature human milk by infants was determined to range between 197 and 770 mg/kg body weight/day, with a maximum intake of up to 1,254 mg/kg body weight/day among infants from Secretor mothers. In newborns, intake was derived in GRN 546 assuming a body weight of 3.4 kg (WHO, 2015), and a milk consumption of 250 mL/day during the first 5 days postpartum (Hester *et al.*, 2012). The average intake of 2'-FL from colostrum was estimated to range between 80 and 360 mg/kg body weight/day, with a maximum intake of up to 620 mg/kg body weight/day in newborns from Secretor mothers (GRN 546 Glycom A/S, 2014; U.S. FDA, 2015a).

Kyowa has stated that 2'-FL is intended to be substitutional to other sources of 2'-FL currently on the U.S. market. 2'-FL is currently permitted for use in term (non-exempt) infant formula, hypoallergenic (exempt) infant formula, and toddler formula, infant and toddler foods, and in specified conventional food products and foods for special dietary uses (GRN 546, 571, 650, 735, 749, 815, 852, 897, and 929) (U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a,b, 2020b, 2021). The maximum level of use of 2'-FL permitted in term infant formula and toddler formula (2.4 g/L) was determined in GRN 546 based on the overall mean concentration of 2'-FL in mature milk calculated across studies (U.S. FDA, 2015a). Kyowa proposes to use 2'-FL in food uses currently permitted for other 2'-FL ingredients, as well as additional uses, including breads and baked goods (all varieties), protein drinks, chewing gum, non-dairy cream, non-dairy frozen desserts, edible ices, sherbet and sorbet, energy and protein bars, evaporated and condensed milk, canned fruit, fruit-based desserts, and foods for special dietary uses (*i.e.*, oral nutritional supplements and enteral tube feeding formula). All proposed conditions of use of 2'-FL are presented in Table A-2 of Attachment A.

Dietary exposure to 2'-FL was assessed using food consumption data available in the 2017-2018 cycle of the U.S. National Health and Nutrition Examination Survey (CDC, 2021a,b; USDA, 2021). The GRAS Panel reviewed dietary exposure estimates of 2'-FL considering all proposed conditions of use in various U.S. population groups, estimates from conditions of use in term infant formula and toddler formula only in infant and toddler population groups, and estimates from consumption of food for special dietary uses only.

Considering all proposed food uses, the resulting consumer-only mean and 90th percentile intakes of 2'-FL by the total U.S. population (≥2 years of age) were estimated to be 9.4 g/person/day (143 mg/kg body weight/day) and 17.4 g/person/day (298 mg/kg body weight/day), respectively. Among the individual population groups, the highest mean and 90th percentile intakes of 2'-FL on an absolute basis were determined to be 11.7 g/person/day (146 mg/kg body weight/day) and 20.6 g/person/day (261 mg/kg body weight/day), respectively, as identified among the elderly. Infants 0 to 6 months of age had the lowest consumer-only intakes on an absolute basis of 2.4 and 4.3 g/day at the mean and 90th percentile, respectively, while infants 7 to <12 months had the highest daily mean and 90th percentile intakes on a body weight basis, of up to 520 mg/kg body weight/day (4.7 g/person/day) and 886 mg/kg body weight/day (8.0 g/person/day), respectively. The mean and 90th percentile consumer-only intakes of 2'-FL from use in infant formulas and toddler formula only were highest in infants 0 to 6 months of age on both an absolute and body weight basis, at 295 mg/kg body weight/day (1.87 g/person/day) and 494 mg/kg body weight/day (2.96 g/person/day), respectively.

[1] https://www.cdc.gov/growthcharts/who_charts.htm (CDC, 2010).

The estimated daily intake of 2'-FL from proposed conditions of use in infants was compared to that from mature human milk. Mean consumer-only intakes from all proposed uses (355 to 520 mg/kg body weight/day) are within the average range of 2'-FL intakes from mature breast milk (197 to 770 mg/kg body weight/day), whereas 90th percentile intakes (570 to 886 mg/kg body weight/day) are below the maximum estimated daily intake of 2'-FL from mature Secretor milk (1,254 mg/kg body weight/day). Considering exposure from infant formulas and toddler formula only, mean and 90th percentile consumer-only intakes of 2'-FL (up to 295 and 494 mg/kg body weight/day, respectively) are within the average range of 2'-FL intakes from mature human milk (197 to 770 mg/kg body weight/day), and below maximum 2'-FL intakes from mature Secretor milk (1,254 mg/kg body weight/day). As 2'-FL intakes from all proposed conditions of use are within background exposure from human milk in infants, a vulnerable population group, 2'-FL is considered to be safe for all population groups.

The GRAS Panel considered additive exposure from complementary foods supplemented with 2'-FL by breastfed infants and noted that breastfed infants are not expected to be high consumers of both 2'-FL from breast milk and 2'-FL from complementary foods, as the highest observed concentration of 2'-FL in breast milk occurred in Secretor milk within 1 month of lactation, at which point infants are unlikely to be exposed to complementary foods. Furthermore, the concentration of 2'-FL in human milk decreases as lactation progresses, and the consumption of breast milk would decrease as the consumption of complementary foods increases. Thus, additive exposure from high-level consumption of 2'-FL from breast milk and high-level consumption of complementary foods was considered unlikely. The GRAS Panel concluded that no safety concerns are anticipated due to consumption of complementary foods supplemented with 2'-FL by breastfed infants.

Under the recommended conditions of use in foods for special dietary uses, use of 2'-FL in oral nutritional supplements for ages 2 and up and enteral tube feeding formula for ages 11 and up would result in total daily intakes of 4 and 10 g 2'-FL/day, respectively, which are less than the highest estimated 90th percentile intakes of 2'-FL from all proposed uses and therefore also below the maximum estimated daily intake of 2'-FL from mature Secretor milk. The GRAS Panel noted that foods for special dietary use containing 2'-FL are not intended to be consumed in combination with any other supplemental sources of 2'-FL and will be labeled as such. Consumption of 2'-FL from foods for special dietary use was therefore concluded to be substitutional and not additive to consumption of 2'-FL from other sources.

As Kyowa's 2'-FL is identical to 2'-FL in human breast milk, the GRAS Panel considered the comparison of intakes from the proposed uses to natural background dietary exposures to 2'-FL from the consumption of human milk to be pivotal in the assessment of the safety of Kyowa's 2'-FL.

DATA PERTAINING TO SAFETY

The safety of Kyowa's 2'-FL ingredient is supported by the results of published safety studies of 2'-FL previously evaluated by various experts qualified by scientific training and experience to evaluate the safety of food ingredients (GRN 546, 571, 650, 735, 749, 815, 852, 897, and 929), as well as by the U.S. FDA (U.S. FDA, 2015a,b, 2016, 2018a,b, 2019a,b, 2020b, 2021) and EFSA (EFSA, 2015). Additional safety studies identified in a comprehensive and detailed search of the published scientific literature (conducted 12 April 2021) were critically reviewed by the GRAS Panel and considered supportive of safety; unpublished safety studies conducted by Kyowa were also critically reviewed by the GRAS Panel and concluded to be corroborative in nature. Consistent with the requirements of the GRAS standard, conclusions on the GRAS status of 2'-FL have considered all publicly available sources of information, including favorable and potentially unfavorable information. Based on Kyowa's search of the literature, the company is not aware of published studies indicating that 2'-FL is unsafe for use as a food ingredient.

Absorption, Distribution, Metabolism, and Excretion

Results from a number of studies have demonstrated that HMOs are resistant to hydrolysis by digestive enzymes in the upper digestive tract (Engfer *et al.*, 2000; Gnoth *et al.*, 2000) and are either partially fermented by the intestinal microbiota or excreted unchanged in the feces (Brand-Miller *et al.*, 1995, 1998; Chaturvedi *et al.*, 2001; Coppa *et al.*, 2001; Albrecht *et al.*, 2011; Kuntz *et al*., 2019). 2'-FL fermentation metabolites have been demonstrated to be absorbed, distributed throughout the body, and excreted in the urine of wild-type but not germ-free mice (Kuntz *et al.*, 2019). Low levels of 2'-FL have been detected in the urine and plasma of breastfed infants (Rudloff *et al.*, 1996, 2012; Obermeier *et al.*, 1999; Chaturvedi *et al.*, 2001; Dotz *et al.*, 2014; Goehring *et al.*, 2014), infants receiving formula supplemented with 2'-FL (Marriage *et al.*, 2015), and rats administered a single oral dose of 2'-FL (Vazquez *et al.*, 2017), indicating that small amounts of 2'-FL are absorbed. 2'-FL absorption profiles were demonstrated to be similar in plasma samples collected from infants fed formulas supplemented with 2'-FL and infants fed human milk (Marriage *et al.*, 2015).

The levels of other carbohydrates in Kyowa's 2'-FL are comparable to those in other 2'-FL ingredients (synthetic and microbial source) which have been concluded to be GRAS and notified to the U.S. FDA. Lactose and fucose are naturally occurring components of human milk, difucosyllactose is a human breakdown product of naturally-occurring fucosylated oligosaccharides (fucosylgalactose), and glucose and galactose are naturally-occurring breakdown products of lactose and are common dietary components. These other carbohydrates will follow well-documented metabolic pathways, with no safety concerns expected due to the low dietary exposure to these carbohydrates from the proposed uses of Kyowa's 2'-FL compared to background dietary exposures.

Toxicological Studies – Kyowa's 2'-FL Ingredient

Genotoxicity

The GRAS Panel critically evaluated the results of a bacterial reverse mutation assay conducted using 2'-FL manufactured by Kyowa and produced by fermentation using a genetically modified strain of *E. coli* W (Oguma, 2019a [unpublished]). The assay was conducted according to Organisation for Economic Co-operation and Development (OECD) Principles of Good Laboratory Practice (GLP) and OECD Test Guideline 471 (OECD, 1997, 1998). In this study, *Salmonella typhimurium* strains TA100, TA1535, TA98, and TA1537 and *E. coli* strain WP2 *uvrA* were incubated with Kyowa's 2'-FL (assay 92%) at concentrations of 0 (water as negative control, or positive controls), 313, 625, 1,250, 2,500, or 5,000 μg/plate, in the presence or absence of metabolic activation. No increase in the number of revertant colonies were observed in the presence or absence of metabolic activation, and the study authors concluded that 2'-FL was not mutagenic under the conditions of this study.

The potential genotoxicity of Kyowa's 2'-FL ingredient (assay 92%) also was evaluated in an *in vivo* micronucleus study that was conducted in accordance with GLP and OECD Test Guideline 474 (OECD, 1998, 2016a; Oguma, 2019b [unpublished]). In this study, male Slc:ICR mice were administered 2'-FL by gavage at doses up to 2,000 mg/kg body weight, with water used as a negative control, and mitomycin C used as a positive control. No statistically significant differences in the frequency of micronucleated immature erythrocytes or the proportion of immature erythrocytes were reported, and the study authors concluded that 2'-FL does not induce chromosomal aberrations in mice.

The GRAS Panel noted that the results of these 2 studies are consistent with those reported by other investigators and further corroborate the conclusion that 2'-FL is not genotoxic.

Subchronic Toxicity Studies

The potential toxicity of Kyowa's 2'-FL ingredient was evaluated in a 90-day toxicity study conducted in accordance with OECD Test Guideline 408 and GLP (OECD, 1998, 2018; Tsuboi, 2020 [unpublished]). In this study, 6-week-old Crl:CD(SD) rats (10/sex/group) were administered Kyowa's 2'-FL (assay 92%) in distilled water by gavage at doses of 0 (distilled water), 500, 1,000, or 2,000 mg/kg body weight/day. A battery of parameters relevant to safety were assessed, including mortality, body weight, food consumption, clinical signs, functional observation, organ weights, gross and histological pathology, ophthalmology, clinical chemistry, and hematology. No toxicologically relevant compound-related adverse effects were reported, and the study authors determined the no-observed-adverse-effect level (NOAEL) to be 2,000 mg/kg body weight/day, the highest dose tested. The GRAS Panel noted that the results of this study are consistent with the published literature and concluded that the results of this study corroborate the safety of Kyowa's 2'-FL ingredient.

Toxicological Studies – Other 2'-FL Ingredients

Acute Studies

The GRAS Panel critically evaluated a single dose oral toxicity study of 2'-FL manufactured by Kyowa and produced by a fermentation method different from that described above (*i.e.*, including the use of different genetically modified strains of *E.t coli* W) (BoZo Research Center Inc., 2016a [unpublished]). The 2'-FL test article was reported to contain 95.3% 2'-FL (100% purity on a dry weight basis) and 4.9% water. 2'-FL was administered at a dose of 5,000 mg/kg body weight to Sprague-Dawley rats (5/sex/group) at 6 weeks of age following an overnight fast. No clinical signs, gross pathological abnormalities, adverse effects on body weight, or mortalities were reported over the course of a 14-day observation period. The GRAS Panel considers acute toxicity studies to be of limited value to the safety assessment of a food ingredient; this information is therefore corroborative in nature.

Subchronic and Chronic Studies

A number of repeat-dose toxicity studies assessing the safety and tolerability of 2'-FL produced by chemical synthesis or by microbial fermentation, or mixtures of HMOs or poorly-digestible carbohydrates including 2'-FL, conducted in neonatal, juvenile, and adult rats, have been comprehensively reviewed in previous GRAS Notices of 2'-FL and during novel food evaluations by EFSA (Jennewein Biotechnologie, 2013, 2014 [unpublished]; Coulet *et al.*, 2014; Penard, 2015 [unpublished]; Glycosyn, LLC and Friesland Campina Domo B.V., 2016, 2017a,b [unpublished] in Glycosyn, LLC and Friesland Campina Domo B.V., 2018; van Berlo *et al.*, 2018).

The lowest author-reported 90-day NOAEL of 2'-FL is 5,000 mg/kg body weight/day, which was reported in 2 studies in rats (Coulet *et al.*, 2014; Penard, 2015 [unpublished]). The EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) evaluated the study by Coulet *et al.* (2014) and established a NOAEL of 2,000 mg/kg body weight/day, the lowest dose tested. However, the EFSA NDA Panel based their conclusions on findings that were determined by the study authors to be non-adverse, not related to the treatment, or of no biological or toxicological significance. In previous evaluations by various experts qualified by scientific training and experience to evaluate the safety of food ingredients and by the U.S. FDA, it has been unanimously concluded that the study authors' NOAEL determinations were appropriate (Glycom A/S, 2018). Based on the totality of information characterizing the toxicity of 2'-FL in rodents that has been published to date, the GRAS Panel agreed that a NOAEL of 5,000 mg 2'-FL/kg body weight/day (Coulet *et al.*, 2014) was supported.

The GRAS Panel critically evaluated the results of an additional 90-day study of a product containing 31.5% 2'-FL and 59.4% lacto-*N*-fucopentaose I (LNFP-I; produced by Glycom A/S *via* fermentation) (Phipps *et al.*, 2020). This study was conducted in accordance with GLP and OECD Test Guideline 408 (OECD, 1998, 2018) (with the exception that the animals were 7 days of age at the beginning of the dosing period) to evaluate the safety of the product in neonatal rats. Neonatal Sprague-Dawley rat pups were administered 2'-FL/LNFP-I at doses of 0 (water), 1,000, 3,000, or 5,000 mg/kg body weight/day by gavage for 90 days. The low- and mid-dose groups comprised 10 pups/sex/dose, while the control and high-dose groups comprised 15 pups/sex/dose. An additional group (15 pups/sex/dose) was administered 5,000 mg oligofructose/kg body weight/day for 90 days as a reference control. Recovery groups (5 pups/sex/dose) were retained for a 4-week period following administration of vehicle control, 5,000 mg oligofructose/kg body weight/day, or 5,000 mg 2'-FL/LNFP-I/kg body weight/day for 90 days. No test item-related adverse effects were reported with respect to clinical signs, body weight, food consumption, time to sexual maturity, behavior, estrous cycles, organ weights, gross or histopathology, or hematology, clinical biochemistry, or urinalysis parameters. The study authors therefore concluded the NOAEL to be 5,000 mg 2'-FL/LNFP-I/kg body weight/day (the highest dose tested, which provided 1,575 mg 2'-FL/kg body weight/day) and noted that the results support the use of these compounds in infant formula and foods for the general population.

The GRAS Panel critically evaluated the results of 2 newly identified studies. In the first study, 2'-FL produced by microbial fermentation (>90% purity; production strain not reported) was administered daily by gavage for 14 days to 2-day-old Lewis rats (LEW/OrlRj) at a dose of 0 (water vehicle control) or 2,000 mg/kg body weight/day (Azagra-Boronat *et al.*, 2019). Primary endpoints were related to gut immunity and the gut microbiome, though several safety parameters were evaluated including body weight, body-to-tail-length ratio, relative organ weights, and fecal characteristics. Compared to controls, rats from the 2'-FL group had a slightly higher body weight at Day 16, and a higher body-to-tail-length ratio at Days 8 and 16, though the study authors concluded that differences in growth were not biologically relevant. The relative weight of the large intestine was slightly decreased at Day 16 in rats treated with 2'-FL compared to controls, likely due to the increased body weight reported in this group, which was determined to be non-biologically relevant. The study authors concluded that 2'-FL was safe and well-tolerated as relative organ weights and stool characteristics were not affected by 2'-FL supplementation.

In the second study, CD/Crl:CD rats were fed diets containing 0 or 10% HMO MIX I in a 7-day oral tolerance study and in a 13-week oral toxicity study (Parschat *et al.*, 2020). The HMO MIX I was composed of 5 HMOs, including 2'-FL, which was the most abundant HMO of the mix (2'-FL: 47.1% dry weight; 3-fucosyllactose: 16.0% dry weight; lacto-*N*-tetraose: 23.7% dry weight; 3'-sialyllactose: 4.1% dry weight; and 6'-sialyllactose: 4.0% dry weight; other carbohydrates: 5.1% dry weight). In both the 7-day oral tolerance study and 13-week oral toxicity study, no treatment-related effects were reported. Based on the results of the 13 week study, the study authors concluded that the NOAEL of the HMO MIX I was 10% in the diet, the only concentration investigated, equivalent to 5.67 and 6.97 g HMO MIX I/kg body weight/day (2.67 and 3.28 g 2'-FL/kg body weight/day) in males and females, respectively. It is noted that these are single dose studies and thus are of limited value.

Neonatal Piglet Study

A study in which the tolerance and safety of the supplementation of 2'-FL in the diet of domestic Yorkshire Crossbred piglets was evaluated and has been comprehensively reviewed in Section 6.3.3.3 of GRN 571 (Hanlon and Thorsrud, 2014; U.S. FDA, 2015b). Briefly, piglets (4 to 8/sex/group) received liquid diets supplemented with 2'tFL at doses of 0, 200, 500, or 2,000 mg/L from lactation Day 2 for a duration of 3 weeks. The study authors concluded that 2'-FL supplementation at up to 2,000 mg/L (equivalent to 291.74 and 298.99 mg/kg body weight/day in males and females, respectively) was well-tolerated in

neonatal piglets. Furthermore, as there were no treatment-related effects on growth and development nor on clinical pathology and histopathological parameters, the study authors concluded that 2'-FL supplementation at the concentrations evaluated was not associated with any adverse effects and did not impact growth. As such, the NOAEL from this 3-week dietary study in neonatal piglets was concluded to be 291.74 mg 2'-FL/kg body weight/day.

Genotoxicity Studies

The genotoxicity of 2'-FL produced by chemical synthesis or by microbial fermentation has been previously evaluated in bacterial reverse mutation assays and either an *in vitro* mammalian cell gene mutation test in cultured mouse lymphoma L5178Y cells (GRN 546) (U.S. FDA, 2015a), an *in vitro* mammalian cell micronucleus test in cultured peripheral human lymphocytes (GRN 650, 735, and 815) (U.S. FDA, 2016, 2018a, 2019a), or an *in vivo* mammalian micronucleus test in CD Crl: CD(SD) rats (GRN 571) (U.S. FDA, 2015b), all of which were conducted according to applicable test guidelines of the OECD. In all studies, 2'-FL was concluded to be non-mutagenic and non-genotoxic under the conditions of the assays.

The mutagenicity of another 2'-FL ingredient (purity 95.3% w/w; water 4.9% w/w) manufactured by Kyowa using modified strains of *E. coli* W (different from the production strain that is the subject of the current GRAS assessment) was evaluated in a bacterial reverse mutation assay conducted according to OECD Test Guideline 471 (OECD, 1997) (Bozo Research Center Inc., 2016b [unpublished]). Negative results were reported in *S. typhimurium* strains TA100, TA1535, TA98, and TA1537 and in *E. coli* WP2 *uvrA*, with and without metabolic activation, at concentrations up to 5,000 µg/plate, and the study authors concluded that 2'-FL was non-mutagenic under the conditions of the assay. The GRAS Panel noted that these findings are consistent with those reported by other investigators and therefore further corroborate the conclusion that 2'-FL is not mutagenic.

The GRAS Panel reviewed the results of a bacterial reverse mutation assay and an *in vitro* mammalian cell micronucleus assay in which the genotoxic potential of HMO MIX I was evaluated according to OECD Principles of GLP (OECD, 1998) and OECD Test Guidelines 471 (OECD, 1997) and 487 (OECD, 2016b), respectively (Parschat *et al.*, 2020). As per the subchronic toxicity studies of HMO MIX I, the mix was composed of 5 HMOs, including 2'-FL, which was the most abundant HMO of the mix (2'-FL: 47.1% dry weight; 3-fucosyllactose: 16.0% dry weight; lacto-N-tetraose: 23.7% dry weight; 3'-sialyllactose: 4.1% dry weight; and 6'-sialyllactose: 4.0% dry weight; other carbohydrates: 5.1% dry weight). Mutagenic potential was assessed in various strains of *S. typhimurium* (TA98, TA100, TA102, TA1535, and TA1537) and in *E. coli* WP2 *uvrA* at concentrations up to 600 mg/plate (equivalent to up to 283 mg 2'-FL/plate), with and without metabolic activation, using pre-incorporation and pre-incubation methods. Genotoxic potential was assessed in cultured peripheral human lymphocytes at concentrations up to 60 mg/mL (equivalent to up to 28 mg 2'-FL/mL), with and without metabolic activation, at 4- and 24-hour exposure times. The HMO MIX I was concluded to be non-mutagenic and non-genotoxic under the conditions of the assays.

Two additional genotoxicity studies of the mixed product containing 31.5% 2'-FL and 59.4% LNFP-I (produced by Glycom A/S *via* fermentation) identified in the recently published literature were reviewed by the GRAS Panel, including a bacterial reverse mutation assay conducted in compliance with OECD Test Guideline 471 (OECD, 1997) and an *in vitro* micronucleus assay conducted in compliance with OECD Test Guideline 487 (OECD, 2016b). In the former study, negative results were reported in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 *uvr*A (with or without metabolic activation) when tested according to the plate incorporation or pre-incubation methods at concentrations up to 5,000 µg/plate (Phipps *et al.*, 2020). In the latter study, negative results were reported upon incubation of

cultured human peripheral lymphocytes with the same test item at concentrations up to 2,000 µg/mL with or without metabolic activation (Phipps *et al.*, 2020).

The GRAS Panel noted that the results of the genotoxicity studies reviewed, in addition to the natural occurrence of 2'-FL as a component of human breast milk, demonstrate that 2'-FL lacks genotoxic potential.

Clinical Studies

Studies in Infants

The GRAS Panel noted that the safety and tolerance of the supplementation of 2'-FL.in infant formula alone or in combination with lacto-*N*-neotetraose (LNnT) or non-milk oligosaccharides [galactooligosaccharides (GOS) or short-chain fructooligosaccharides (scFOS)] has been previously evaluated in a number of clinical studies conducted in full-term infants 0 to 6 months of age (Marriage *et al.*, 2015; Goehring *et al.*, 2016; Kajzer *et al.*, 2016; Puccio *et al.*, 2017; Storm *et al.*, 2019). The authors of these studies consistently concluded that 2'-FL supplementation at levels ranging from 0.2 to 1.0 g/L was safe and well-tolerated in young infants.

The GRAS Panel reviewed 4 new intervention studies of 2'-FL conducted in infants, which were not included in previous GRAS evaluations submitted to the U.S. FDA or evaluated by EFSA, and which were identified in a comprehensive search of the published scientific literature conducted on 12 April 2021. In these studies, healthy, term infants or toddlers were fed formula containing 0.25 to 3 g 2'-FL/L in addition to milk fats/proteins, probiotic bacteria, and/or poorly digestible carbohydrates for 6 weeks to 6 months (Storm *et al.*, 2019; Leung *et al.*, 2020; Román Riechmann *et al.*, 2020; Vandenplas *et al.*, 2020). Although some potentially test formula-related minor adverse events were reported (*i.e.*, cow's milk intolerance and irritability), there were no significant differences in the incidences of adverse events and the authors of all 4 studies concluded that the formulas were well tolerated, with no safety concerns noted.

Vandenplas *et al.* (2020) reported no test formula-related effects on growth, adverse events, serious adverse events, or gastrointestinal tolerance in infants fed cow's milk formula supplemented with 2'-FL (1 g/L), short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) (9:1; 8 g/L), and milk fat (49.8% of total fat) for 15 weeks compared to those fed a standard cow's milk formula. Likewise, Román Riechmann *et al.* (2020) reported no test formula-related adverse effects on growth, gastrointestinal tolerance, or adverse events in infants fed partially hydrolyzed whey-based formula supplemented with 1 g 2'-FL/L, 0.5 g LNnT/L, and *Lactobacillus reuteri* for 8 weeks compared to a reference group of exclusively breastfed infants or a group fed with breast milk and infant formula.

Leung *et al.* (2020) reported longer (but not more frequent) upper respiratory tract infections, more incidences of cough and runny nose, more days with fever, and more gastrointestinal infection episodes in children 1 to 2.5 years of age consuming formula supplemented with 2'-FL (3 g/L) with or without additional milk fat and bioactive proteins for 6 months compared to those fed a standard milk formula. However, the authors reported that there were no between-group differences in the incidence of adverse events or serious adverse events, with none of the reported events considered to be product-related. Storm *et al.* (2019) reported soft or loose stool in 77% of infants fed formula containing *Bifidobacterium lactis* with 0.25 g 2'-FL/L for 6 weeks, as well as a significant increase in the number of infants who spit up >5 times/day, compared to those fed formula supplemented with *B. lactis* only. However, no significant difference in the number of adverse events between groups was reported, and the authors concluded that no safety concerns were identified and the test formulas were well tolerated. Notably, increased incidence

of loose stools and spitting up were not reported in other studies in which infants or toddlers were fed formula containing higher concentrations of 2'-FL alone or with other infant formula ingredients.

Studies in Infants with Food Allergy and/or Feeding Intolerance

In addition to intervention studies in healthy term infants, studies in term infants with cow's milk protein allergy (CMPA), suspected food protein allergy, persistent feeding intolerance, or other conditions warranting the use of extensively hydrolyzed infant formula, were identified and are summarized below. The results of these studies provide support for the suitability and safety of the use of 2'-FL as an ingredient in hypoallergenic infant formula.

The GRAS Panel critically evaluated a clinical study assessing the allergenic potential, tolerability, and safety of a whey-based extensively hydrolyzed formula (EHF) supplemented with 2'-FL (1.0 g/L) and LNnT (0.5 g/L) in infants and children 2 months to 4 years of age with cow's milk protein allergy (CMPA) (Nowak-Wegrzyn *et al.*, 2019). The risk of hypersensitivity was evaluated in a crossover double-blind placebo-controlled food challenge, where the placebo control formula was a commercially available hypoallergenic EHF without HMOs (Althéra®, Nestlé Health Science, Vevey, Switzerland). The sample size was calculated to meet the American Academy of Pediatrics (AAP) criteria for assessing hypoallergenicity of infant formulas, where, at minimum, it must be demonstrated with 95% confidence that 90% [95% lower bound confidence interval (CI) ≥90%] of infants with documented CMPA will not react with defined symptoms (AAP, 2000). Following an initial lip dose challenge, oral doses of the assigned EHF were administered at 10- to 15-minute intervals providing a total volume of 180 mL (subjects ≤1 year of age) or 240 mL (subjects >2 years of age). The alternate EHF was administered 2 to 7 days later. Subjects were observed for a 1-hour period post-administration, where allergic signs or symptoms were documented and assessed according to pre-defined pass/fail criteria. An open challenge during a period of 7 to 9 days was also conducted, as recommended by the AAP to detect late-onset reactions, during which allergic symptoms, clinical parameters, and adverse events were recorded. The study authors reported 1 allergic reaction to the test formula and 1 allergic reaction to the control formula in both the modified intention-to-treat cohort (n = 63 of 64; 98.4%; 95% CI lower bound 92.8%) and the per protocol cohort (n = 60 of 61; 98.4%; 95% CI lower bound 92.5%). No treatment-related gastrointestinal symptoms or adverse events were reported. The study authors concluded that the hypoallergenicity of the EHF supplement with 2'-FL and LNnT was confirmed according to AAP criteria.

Ramirez-Farias *et al.* (2021) conducted a Good Clinical Practice (GCP)-compliant multicenter study in 47 infants (0 to 60 days of age) with suspected food protein allergy, persistent feeding intolerance, or other conditions warranting the use of extensively hydrolyzed infant formula. All infants were administered formula containing 2'-FL (0 or 0.2 g/L; source not reported) for 2 months, with 36 of the 48 enrolled infants completing the study. Measures of growth as well as daily formula intake, stool observations, and adverse events were recorded throughout the study. No adverse effects were reported with respect to growth or between-group differences in the incidence of adverse effects, and the study authors concluded that the formula containing 2'-FL was well tolerated and safe.

Studies in Adults

The safety and tolerance of 2'-FL, LNnT, and a mixture of the 2 HMOs (2:1 ratio of 2'-FL: LNnT) at levels of up to 20 g/day following 2-week supplementation in adults has also been evaluated (Elison *et al.*, 2016). Increased gastrointestinal symptoms were reported for individuals consuming 20 g 2'-FL/day compared to placebo (nausea, rumbling, bloating, passing of gas, diarrhea, loose stools and urgency to pass stools), though the study authors noted that scores remained low and were rated as mild discomfort, and that it was difficult to determine whether gastrointestinal symptoms were related to the test product, day-to-day variation, or increased awareness by the study participants. A slight but significant increase in the average number of daily bowel movements and in stool consistency was reported in individuals consuming 20 g 2'-FL/day at the end-of-intervention compared to baseline, but was determined by the study authors to be clinically irrelevant. Therefore, it was concluded that 2'-FL was safe and well-tolerated at doses up to 20 g/day.

The GRAS Panel considered an additional study in adults. In this randomized, double-blind, placebo-controlled study, 60 Swedish adults with moderate irritable bowel syndrome (IBS) were assigned to consume a glucose placebo or 5 or 10 g/day of a 4:1 mixture of 2'-FL and LNnT (Glycom A/S) for 4 weeks, followed by a 4-week follow-up period (Iribarren *et al.*, 2020). IBS symptom severity, bowel habits, anxiety, and depression were assessed at baseline and at the end of Study Weeks 4 and 8. Two subjects withdrew from the study due to worsening of IBS symptoms, including 1 subject from each of the placebo and 10 g/day groups. No adverse effects on fecal microbiota were reported, and no between-group differences in severity of overall or individual gastrointestinal symptoms or symptom deterioration were reported. The study authors concluded that the study products were well tolerated and that gastrointestinal symptoms in adult IBS patients were not aggravated by consumption of 10 g of a 4:1 mixture of 2'-FL and LNnT/day for 4 weeks.

Observational Studies in Infants

The GRAS Panel critically evaluated 5 observational cohort studies in which the relationship between HMO composition in human milk and infant growth parameters was examined in infant-mother pairs (Alderete *et al.*, 2015; Sprenger *et al.*, 2017; Berger *et al.*, 2019; Larsson *et al.*, 2019; Lagström *et al.*, 2020), and in 2 of which the relationship to infant body composition was also examined (Alderete *et al.*, 2015; Larsson *et al.*, 2019). The purpose of these studies was to determine whether HMO composition of human milk influences excessive weight gain that has been reported in some breastfed infants. No significant associations were reported in 3 of the 5 studies evaluating growth parameters, as well as in 1 of 2 studies evaluating body composition. In Lagström *et al.* (2020), positive correlations between the concentration of 2'-FL in Secretor milk (collected 3 months postpartum) and height z scores as well as weight z scores were reported in infants 3 to 12 months of age. The study authors noted several limitations, including the collection of milk samples at a single time point even though HMO composition varies throughout lactation, data were unavailable for potential confounding factors (maternal diet and infant morbidity), not all infants were exclusively breastfed and the median duration of breastfeeding was 10 months, and associations reported in infants receiving Secretor milk only could reflect the higher number of Secretor (n = 674) *versus* non-Secretor (n = 100) mothers in the study. In Larsson *et al.* (2019), positive correlations between the concentration of 2'-FL in Secretor milk (collected 5 to 6.5 months postpartum) and weight velocity (g per week) from 0 to 5 months of age, as well as fat mass index at 5 months of age were reported. Several limitations were noted by the study authors, including a small sample size, the lack of adjustment for multiple comparisons and for other milk components in statistical analyses, and the possible introduction of complementary foods. Notably, associations between HMO milk composition and growth were based on milk samples collected at a single timepoint and do not reflect variations in HMO milk composition

throughout the period of excessive weight gain, and no significant difference was reported in the absolute intake of 2'-FL from a 24-hour milk volume at 5 months in infants with high compared to normal weight gain receiving Secretor milk. The study authors from both studies acknowledged that these preliminary exploratory studies do not provide evidence of a causal relationship between HMO composition of human milk and infant weight parameters or body composition. The GRAS Panel noted that there is no conclusive evidence to suggest a concern for excessive weight gain with high concentrations of 2'-FL in breast milk (average or median of 3 to 4 g/L) and no evidence to suggest a safety concern with the intended use level of 2 to 2.4 g 2'-FL/L in infant formula.

Safety of 2'-FL in Enteral Tube Feeding Formula

The GRAS Panel reviewed information relevant to the use of 2'-FL as an ingredient in enteral tube feeding formula at levels up to 20 g/L (intended for patients 11 years of age or older). This use was previously concluded to be GRAS and was notified to the U.S. FDA in GRN 897 (U.S. FDA, 2020b), with safety supported by (i) the low estimated consumption on a body weight basis in comparison to consumption via infant formula, (ii) the lack of genotoxicity and systemic toxicity in high-quality published studies of 2'-FL, with reported NOAELs approximately 150 times higher than expected intake from enteral tube feeding formula [*i.e.*, 5.0 to 7.76 g 2'-FL/kg body weight/day in rodents; the highest doses tested) (Phipps *et al.*, 2018; van Berlo *et al.*, 2018)], and (iii) a lack of compound-related adverse effects reported in high-quality clinical studies of 2'-FL (Kajzer *et al.*, 2016; Nowak-Wegrzyn *et al.*, 2019).

The GRAS Panel critically reviewed data submitted to the U.S. FDA in response to questions on GRN 897, including the results of 19 published studies of the safety/tolerability of other poorly-digestible carbohydrates as components of enteral tube feeding formula (at doses up to 63 g/day) that were considered in lieu of relevant safety or tolerability studies of 2'-FL. The GRAS Panel concurred with the notifier's conclusion that the safety of the use of 2'-FL as an ingredient in enteral tube-feeding formula at levels up to 20 g/kg is supported by the lack of test compound-related adverse effects reported in these 19 studies, as well as the Institute of Medicine's conclusion that establishing a tolerable upper intake level for fiber is not necessary (U.S. FDA, 2020b). The GRAS Panel noted that upon consideration of the information provided by the notifier, the U.S. FDA responded with no questions regarding the GRAS status of 2'-FL under the conditions of use specified in GRN 897, including use in enteral tube feeding formula at levels up to 20 g/L.

The GRAS Panel concluded that the safety of 2'-FL in formula for enteral tube feeding at a use level of 20 g/L is supported by the safety profile of the ingredient and the safety of poorly-digestible carbohydrates in general in enteral feeding at levels that exceed the recommended intake of 2'-FL from the intended use in formula for enteral tube feeding.

Allergenicity

Possible transfer of protein originating from the fermentation broth is controlled during the manufacturing process through the removal of production organism from the fermentation media and through downstream processing of the media during the purification process. The GRAS Panel noted that analytical data demonstrating the absence of the production organism and production organism-derived DNA in the final 2'-FL ingredient support the effective removal of these potential impurities from the final ingredient. The GRAS Panel also noted that the purification processes have been demonstrated to remove residual protein to a level that is well below Kyowa's specification for residual protein (10 mg/kg) and below the limit of detection of 1 mg/kg (0.0001%) using dot blot analysis. The results of analysis of the final 2'-FL ingredient using 2 enzyme-linked immunosorbent assay (ELISA) test kits [FASPEK ELISA II Milk (Casein; Morinaga Institute of Biological Science, Inc.) and FASTKIT ELISA Ver. III MILK (NH Foods Ltd.)], with quantification limits of 1.0 μg/g, demonstrate that milk proteins are effectively removed during the purification process and are not present in Kyowa's final 2'-FL ingredient. In addition, no published reports of sensitization, case reports of allergic reactions, or allergenicity studies on 2'-FL were identified in a comprehensive and detailed search of the published scientific literature that was conducted on 12 April 2021 to identify studies relevant to the safety of 2'-FL. The GRAS Panel considered Kyowa's 2'-FL manufactured with a genetically modified strain of *E. coli* W to be of low allergenic risk and noted that the low allergenic risk of Kyowa's 2'-FL supports its safe addition to exempt hypoallergenic infant formula in the U.S.

CONCLUSION

We, the undersigned, independent, qualified members of the Generally Recognized as Safe (GRAS) Panel, have independently and collectively, critically evaluated the data and information summarized above that is pertinent to the safety of the proposed use of 2'-FL. We unanimously conclude that the proposed uses in infant formula, conventional foods, and foods for special dietary uses specified herein of Kyowa's 2'-FL produced by microbial fermentation by a genetically modified strain of *E. coli* W, meeting appropriate food grade specifications and produced in accordance with current good manufacturing practice, are GRAS based on scientific procedures.

It is our professional opinion that other qualified experts would concur with this conclusion.

[redacted]

Virginia Commonwealth University School of Medicine

14 July 2021

Date

Professor Emeritus Robert J. Nicolosi, Ph.D.
University of Massachusetts Lowell

Date

Prof Emeritus Steve L. Taylor, Ph.D.
University of Nebraska-Lincoln

Date

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07/14/2021

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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Last Amended	Section Title
117—Current good manufacturing practice, hazard analysis, and risk-based preventive controls for human food	117	5-1-20	[full section]
130 to 169 [Food Standards]	—	5-1-20	—
170—Food additives	170.3	4-1-19	Definitions

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ATTACHMENT A:
Specifications and Intended Conditions of Use

Table A-1 Chemical and Microbiological Specifications for 2'-FL

Specification Parameter	Specification	Method
Organoleptic		
Appearance	Powder	Visual observation
Color	White to off-white	General Notice, JP[a]
Physicochemical		
Identification	RT of standard ± 3%	HPLC-PAD (internal method)
Purity	≥82% dry basis	HPLC-PAD (internal method)
Water	≤9.0av/w%	JP 2.48[a]
Ash	≤0.5av/w%	JP 2.44[a]
Residual protein	≤10 mg/kg (0.001%)	Dot-blot (internal method)
pH (20°C, 5% solution)	4.0 to 9.0	JP 2.54[a]
Other Carbohydrates		
D-lactose	≤5av/w%	HPLC-PAD (internal method)
L-fucose	≤1av/w%	HPLC-PAD (internal method)
D-glucose and D-galactose	≤1av/w%	HPLC-PAD (internal method)
Difucosyllactose	≤3av/w%	HPLC-PAD (internal method)
Heavy Metals		
Arsenic	≤0.2 mg/kg	USP 233[b]
Cadmium	≤0.2 mg/kg	USP 233[b]
Lead	≤0.2 mg/kg	USP 233[b]
Mercury	≤0.2 mg/kg	USP 233[b]
Iron	≤10 mg/kg	USP 233[b]
Microbiological		
Aerobic plate count	≤1,000 CFU/g	ISO 4833-1:2013
Molds	≤100 CFU/g	ISO 21527-2:2008
Yeasts	≤100 CFU/g	ISO 21527-2:2008
Salmonella	Negative in 100 g	ISO 6579-1:2017
Enterobacteriaceae	Negative in 10 g	ISO 21528-1:2017
Cronobacter spp. (Enterobacter sakazakii)	Negative in 100 g	ISO 22964:2017
Listeria monocytogenes	Negative in 25 g	ISO 11290-1:2017
Bacillus cereus	≤50 CFU/g	ISO 7932:2004
Residual endotoxins	≤10 EU/mg	JP 4.01 (kinetic-turbidimetric method)[a]

2'-FL = 2'-fucosyllactose; CFU = colony forming units; EU = endotoxin units; HPLC-PAD = high-performance liquid chromatography with pulsed amperometric detection; ISO= International Organization for Standardization; JP = Japanese Pharmacopoeia; RT= retention time; USP= United States Pharmacopoeia.

[a] Method is consistent with the compendial method specified in 17th edition of the Japanese Pharmacopeia (2016).

[b] Method is consistent with the compendial method specified in the United States Pharmacopeia 35th revision (2011).

Table A-2 Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the United States

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Food Uses[a,b]	Use Levels (g/L or g/kg)
Baked Goods and Baking Mixes	**Breads and baked goods**[c], incl. gluten-free	24 to 48
Beverages and Beverage Bases	Soft drinks (regular and diet)	1.2
	Enhanced, fortified, and flavored waters (incl. carbonated waters)	1.2
	Non-milk-based meal replacement drinks for weight reduction	5
	Sports, isotonic, and energy drinks	0.8 to 1.2
	Protein drinks	**5**
Breakfast Cereals	Hot breakfast cereals (*e.g.*, oatmeal, grits), instant and RTE	31
	RTE breakfast cereals	
	Puffed cereals	80
	High-fiber cereals	30
	Biscuit-type cereals	20
Chewing Gum	**Chewing gum**	**300**
Coffee and Tea	Coffee	5.0 to 10.0
	Tea	5.0 to 10.0
Dairy Product Analogs	Milk substitutes such as soy milk and imitation milks	1.2
	Beverage whiteners	600
	Non-dairy cream	**80**
	Non-dairy yogurt	12
	Non-dairy frozen desserts	**17**
Frozen Dairy Desserts and Mixes	Frozen desserts incl. ice creams and frozen yogurts, frozen novelties	17
Fruit and Water Ices	**Edible Ices, Sherbet, and Sorbet**	**17**
Gelatins, Puddings, and Fillings	Dairy-based puddings, custards, and mousses[d]	17
	Fruit pie filling	14.1
	"Fruit Prep" such as fruit filling in bars, cookies, yogurt, and cakes	30
Grain Products and Pastas	Cereal and granola bars incl. **energy, protein**, and meal replacement bars[e]	40
Infant and Toddler Foods	Term infant formula	2.4 (as consumed)
	Toddler formula	2.4 (as consumed)
	Hypoallergenic infant formula[f]	2.4 (as consumed)
	Other baby foods for infants and young children	12
	Hot cereals (dry and RTE)	10.9
	Other drinks for young children, incl. yogurt and juice beverages identified as "baby drinks"	1.2 to 10
	Desserts incl. fruit desserts, cobblers, yogurt/fruit combinations ("junior type desserts")	10.9
	Baby crackers, pretzels, cookies, and snack items	57
Jams and Jellies	Jellies and jams, fruit preserves, and fruit butters	60
Milk, Whole, and Skim	Unflavored pasteurized and sterilized milk (whole milk, reduced-fat milk, low-fat milk, non-fat milk; including powdered milks, reconstituted)	1.2

Table A-2 Summary of the Individual Proposed Food Uses and Use Levels for 2'-FL in the United States

Food Category (21 CFR §170.3) (U.S. FDA, 2020a)	Food Uses[a,b]	Use Levels (g/L or g/kg)
Milk Products	Buttermilk	1.2
	Flavored milk	1.2
	Evaporated and condensed milk	**1.2**
	Milk-based meal replacement beverages for weight reduction	1.2 to 5
	Yogurt	12
	Formula intended for pregnant women ("mum" formulas, -9 to 0 months)[g]	6
Processed Fruits and Fruit Juices	Fruit flavored drinks and ades	1.2
	Fruit juices and nectars	1.2
	Canned fruit	**17**
	Fruit-based desserts	**17**
Processed Vegetables and Vegetable Juices	Vegetable juices and nectars	1.2
Sugar Substitutes	Table-top sweeteners	300
Sweet Sauces, Toppings, and Syrups	Syrups used to flavor milk beverages	7
Foods for Special Dietary Uses	Oral nutritional supplements and enteral tube feeding (11 years and older)[h]	20[i]

2'-FL = 2'-fucosyllactose; CFR = *Code of Federal Regulations*; FDA= Food and Drug Administration; GRAS= Generally Recognized as Safe; incl.= including; NHANES = National Health and Nutrition Examination Survey; RTE= ready-to-eat; U.S.= United States.

[a] 2'-FL is intended for use in unstandardized products where standards of identity, as established under 21 CFR §130 to 169, do not permit its addition in standardized products (U.S. FDA, 2020a).

[b] Additional food uses proposed by Kyowa that have not been previously concluded as GRAS and notified to the U.S. FDA are **bolded**.

[c] The use of 2'-FL was previously concluded to be GRAS in gluten-free breads and baked goods. Kyowa now proposes to also use 2'-FL in conventional breads and baked goods (*i.e.*, both gluten containing and gluten-free products).

[d] Includes gelatin desserts.

[e] The maximum use level of 2'-FL previously concluded to be GRAS in cereal and granola bars (excluding **energy, protein**, and meal replacement bars) was 30 g/kg. The maximum use level of 2'-FL previously concluded to be GRAS in meal replacement bars was 40 g/kg.

[f] The use of 2'-FL was previously concluded to be GRAS in hypoallergenic infant formula at a use level of 2.0 g/L.

[g] Food codes for "mum formulas" were not available in the 2017-2018 NHANES. This intended use is excluded from the calculation of estimated daily intakes due to absence of consumption data.

[h] Foods for special dietary use were assessed separately from the intended food uses of 2'-FL in conventional foods, as they are intended for supplying a particular dietary need and/or supplementing the intake of a dietary component. Intake of 2'-FL from foods for special dietary use is, therefore, not expected to be cumulative to other dietary sources.

[i] Use level of 20 g/L represents the level of 2'-FL in the final, ready to consume product.