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March 30, 2020

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

Subject: GRAS Notification –
2'-Fucosyllactose
As a Food Ingredient

Dear Dr. Gaynor,

On behalf of Advanced Protein Technologies Corp. (APTech), we are submitting a GRAS notification for 2'-fucosyllactose (2'-FL) as a food ingredient. The enclosed document provides the notice of a claim that a food ingredient, the 2'-FL, described in the enclosed notification is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be generally recognized as safe (GRAS), based on scientific procedures, as a food ingredient. We believe that this determination and notification are in compliance with Pursuant to 21 C.F.R. Part 170, subpart E.

Please note that this is a resubmission of GRN 859. The manufacturing process described in this notice is different from those described in other GRAS notices. However, the specifications and composition of APTech's 2'-FL are comparable to those presented in other GRAS notices.

We enclose an original copy of this notification and a CD Rom for your review. Please feel free to contact me if additional information or clarification is needed as you proceed with the review. We would appreciate your kind attention to this matter.

Sincerely,

[redacted]

3/30/2020

Susan Cho, Ph.D.
Susanscho1@yahoo.com
Agent for APTech

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF 2'-FUCOSYLLACTOSE AS A FOOD INGREDIENT

Prepared for
Advanced Protein Technologies, Corp. (APTech)

Prepared by:
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PART 1. SIGNED STATEMENTS AND A CERTIFICATION

Pursuant to 21 CFR Part 170, subpart E, Advanced Protein Technologies, Corp. (hereinafter referred to as ‘APTech’) submits a Generally Recognized as Safe (GRAS) notice and claims that the use of 2’-fucosyllactose (2’-FL) in infant formula and selected conventional foods, as described in Parts 2 through 7 of this GRAS notice, is not subject to premarket approval requirements of the FD&C Act based on its conclusion that the substance is GRAS under the conditions of its intended use.

1.A. Name and Address of the Notifier

Contact: Dr. Jong-Won Yoon
Company: Advanced Protein Technologies, Corp.
Address: 59-5 Seogeunnae-gil, Paltan-myeon, Hwasung City, Gyeonggi-do, 18575, Republic of Korea (South Korea)
Tel: 82-31-888-6245

1.B. Common or Trade Name

2’-fucosyllactose (2’-FL)

1.C. Applicable Conditions of Use of the Notified Substance

1.C.1. Foods in Which the Substance is to be Used

The intended use and use levels of 2’-FL are the same as those described in GRN 735 (pages 5 and 29 to 31), except in medical food applications that were withdrawn from this GRAS notice. As shown in Table 1, APTech proposes to use 2’-FL as an ingredient in whey-, milk-, and/or soy-based infant formulas for full term infants, in formulas for toddlers and children aged 12-36 months, and in selected conventional foods. No uses in pre-term infants are proposed at this time. The combined use with other human milk oligosaccharides (HMO) is not proposed at this time. APTech does not intend to use 2’-FL in medical foods.

1.C.2. Levels of Use in Such Foods

As summarized in Table 1, APTech intends to use 2’-FL as an ingredient in the following food categories:

1) whey-, milk-, and/or soy-based, non-exempt infant formulas for term infants at a maximum level of 2.4 g/L of formula as consumed (ready-to-drink or reconstituted formula prepared from powder),
2) formulas for toddlers and children aged 12 to 36 months at a maximum level of 2.4 g/L of formula as consumed (ready-to-drink or reconstituted formula prepared from powder),
3) foods for infants and toddlers at levels of 0.24 - 1.2 g/serving, and

4) the following food categories at levels of 0.28 - 1.2 g/serving: 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 and milk products, processed fruits and fruit juices, and sweet sauces, toppings, and syrups.

Table 1. Proposed Conventional Food Categories and Intended Use of 2’-FL

Proposed Food Category	Food Uses	Maximum Use Level (g/serving)	Serving Size (g or mL)	Maximum Use Level (g/100 g unless noted otherwise)
Beverages and beverage bases	Energy drinks	0.28	360	0.08
	Fitness water and thirst quenchers, sports and isotonic drinks	0.28	360	0.08
Breakfast cereals	Ready-to-eat breakfast cereals for adults and children	1.20	15 (puffed) 40 (high-fiber) 60 (biscuit-types)	8.00 3.00 2.00
	Hot cereals for adults and children	1.20	40 (dry) ~250 prepared	0.48 (as consumed)
Dairy product analogs	Milk substitutes such as soy milk and imitation milks	0.28	240	0.12
Frozen dairy desserts and mixes	Frozen desserts including ice creams and frozen yogurts, frozen novelties	1.20	~70	1.70
Gelatins, puddings, and fillings	Dairy-based puddings, custards, and mousses	1.20	~70	1.70
	Fruit pie filling	1.20	85	1.41
	Fruit filling in bars, cookies, yogurt, and cakes	1.20	~40	3.00
Grain products and pastas	Bar, including snack bars, meal-replacement bars, and breakfast bars	0.48	40	1.20

Jams and jellies, commercial	Jellies and jams, fruit preserves, and fruit butters	1.20	~20	6.00
Milk, whole, and skim	All Acidophilus or fortified milks, non-fat and low-fat fluids, including fluid milk and reconstituted milk powder	0.28	240	0.12
Milk products	Flavored milks, including milk, coffee drinks, cocoa, smoothies (dairy and fruit-based), other fruit and dairy combinations, yogurt drinks, and fermented milk drinks including kefir	0.28	240	0.12
	Milk-based meal replacement beverages or diet beverages	0.28	240	0.12
	Yogurt	1.20	225	0.53
	Formula intended for pregnant women (-9 to 0 months)	1.20	200	0.60
Processed fruits and fruit juices	Fruit drinks, including vitamin and mineral fortified products	0.28	240	0.12
	Fruit juices	0.28	240	0.12
Sweet sauces, toppings, and syrups	Syrups used to flavor milk beverages	0.28	40	0.70
Other Categories				
Non-exempt infant and follow-on formula	Infant formula* (0 to 6 months), including ready-to-drink formula or reconstituted formula prepared from powder		NA	2.4 g/L
	Follow-on formula* (6-12 months), including ready-to-drink formula or formula prepared from powder		NA	2.4 g/L
	Infant meal replacement products	0.24	100	0.24 (400 mg/100 kcal)

Baby foods	Milk formula for toddlers and children aged 12-36 months*		NA	2.4 g/L
	Ready-to-eat, ready-to-serve, hot cereals	1.20	15 (dry) 110 (ready-to-serve)	1.09 (as consumed)
	Yogurt and juice beverages identified as "baby" drinks	1.20	120	1.00
	Desserts including fruit desserts, cobblers, yogurt/fruit combinations ("junior type" desserts)	1.20	110	1.09
	Baby crackers, pretzels, cookies, and snack items	0.40	7	5.70

Adopted from GRN 735 (pages 30 to 31).
*ready-to-drink or reconstituted formulas prepared from powder; NA=not applicable;

1.C.3. Purpose for Which the Substance is Used

2'-FL is intended for use as an ingredient in infant formulas and selected conventional foods. The addition of 2′-FL to term infant formulas is consistent with efforts to produce infant formula that closely matches the nutrient composition of human milk.

1.C.4. Description of the Population Expected to Consume the Substance

The population expected to consume the substance consists of term infants, toddlers, and members of the general population who consume at least one of the products described above.

1.D. Basis for the GRAS Determination

This GRAS conclusion is based on scientific procedures in accordance with 21 CFR 170.30(a) and 170.30(b).

1.E. Availability of Information

The data and information that are the basis for this GRAS conclusion will be made available to FDA upon request by contacting Jong-Won Yoon at APTech at the address above. The data and information will be made available to FDA in a form in accordance with that requested under 21 CFR 170.225(c)(7)(ii)(A) or 21 CFR 170.225(c)(7)(ii)(B).

1.F. Availability of FOIA Exemption

None of the data and information in Parts 2 through 7 of this GRAS notice are exempt from disclosure under the Freedom of Information Act, 5 U.S.C. §552.

1.G. Certification

We certify that, to the best of our knowledge, this GRAS conclusion is based on a complete, representative, and balanced dossier that includes all relevant information, available and obtainable by us, including any favorable or unfavorable information, and pertinent to the evaluation of the safety and GRAS status of the use of APTech’s 2’-FL.

1.H. Name, Position/Title of Responsible Person Who Signs Dossier, and Signature

Name: Dr. Jong-Won Yoon Date: 3/30/2020
Title: Vice president

Address correspondence to
Dr. Jong-Won Yoon
Advanced Protein Technologies, Corp.
Address: 59-5 Seogeunnae-gil, Paltan-myeon, Hwasung City, Gyeonggi-do, 18575, Republic of Korea (South Korea)
Tel: 82-31-888-6245
E mail: jongwon.yoon91@gmail.com or jwyoon@aptech.biz

1.I. FSIS/USDA Statement

APTech does not intend to add 2’-FL to any meat and/or poultry products that come under USDA jurisdiction. Therefore, 21 CFR 170.270 does not apply.

PART 2. IDENTITY, MANUFACTURING, SPECIFICATIONS, AND TECHNICAL EFFECTS

2.A.1. Identity of the Notified Substance

2.A.1.1. Common Name

2’-fucosyllactose or 2’-*O*-fucosyllactose (2’-FL, 2-FL, 2FL)

2.A.1.2. Chemical Names of Main Component

Chemical Name: α-D-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose

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

2.A.1.3. Chemical Abstract Service (CAS) Registry Number

41263-94-9

2.A.1.4. Empirical Formula

$C_{18}H_{32}O_{15}$

2.A.1.5. Structural Formula

2'-FL is a trisaccharide composed of L-fucose and lactose (D-galactose and D-glucose). The monosaccharide L-fucose is linked to the disaccharide D-lactose by an α-(1→2) bond. Figure 1 shows the structure of 2’-FL.

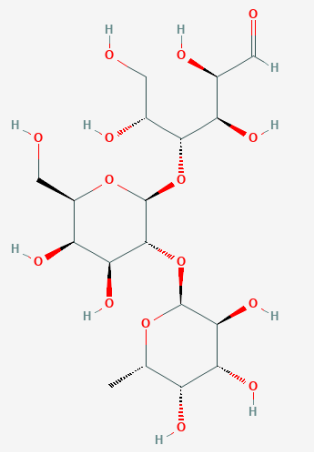

Figure 1. Chemical Structure of 2’-FL

2.A.1.6. Molecular Weight

488.44 daltons

2.A.1.7. Background

2’-FL is a trisaccharide, a type of oligosaccharide, consisting of fucose and lactose (Figure 1). Human milk oligosaccharides (HMOs) all contain lactose at their reducing end. Of the over 200 HMO that have been identified, 2’-FL is the most abundant (Castanys-Munoz et al., 2013). 2’-FL is a HMO that exists in small amounts in beestings (cow’s foremilk), but not in commercialized milk products, whereas it is abundant in human milk.

2.A.2. Potential Toxicants in the Source of the Notified Substance

No toxicant production is expected in the manufacture of 2’-FL. The final product is highly purified through several steps during production.

2.A.3. Particle Size

To check the particle size of APTech’s 2’-FL, 3 non-consecutive batches were analyzed with a LA-950 laser scattering particle size distribution analyzer. The median volume distribution (DV50) had a particle diameter size of 38 - 39 μm (Appendix A).

2.B. Method of Manufacture

The 2’-fucosyllactose (2’-FL) production process by APTech consists of two steps. The first step is the fermentative production of 2’-FL using *Corynebacterium glutamicum* APC199 (a transformant of *Corynebacterium glutamicum* ATCC13032 with the pFP110 plasmid). The components of the fermentation medium are yeast extract, glucose, lactose, and minerals. During the fermentation with the non-pathogenic, non-toxigenic *Corynebacterium glutamicum* APC199 strain, 2’-FL is biosynthesized inside the cells and exported into the culture broth. Upon completion of fermentation, microbial cells are completely removed by micro-filtration systems. Culture supernatant containing 2’-FL is then subjected to downstream purification processes before being dried and packaged.

<u>Details of Biosynthesis of 2’-FL</u>

2’-Fucosyllactose (2’-FL) can be synthesized through the enzymatic transfer of a fucose moiety of GDP-L-fucose to lactose by α-1,2-fucosyltransferase. Additionally, efficient production of GDP-L-fucose and lactose transport are necessary to produce 2’-FL (Figure 2). Our metabolic engineering strategy underlying the construction of the pFP110 plasmid that enables the production of 2’-FL in *Corynebacterium glutamicum* is described below.

Corynebacterium glutamicum has an endogenous metabolic pathway to biosynthesize GDP-D-mannose for producing glycoproteins and glycolipids in cell wall (Jackson and Brennan, 2009; Mishra et al., 2011). Further conversion of GDP-D-mannose into GDP-L-fucose by two heterologous enzymes (GDP-D-mannose-4,6-dehydratase and GDP-L-fucose synthase) is necessary to produce GDP-L-fucose in *C. glutamicum*. Therefore, *gmd* and *wcaG* coding for GDP-D-mannose-4,6-dehydratase and GDP-L-fucose synthase of *Escherichia coli* (*E.coli*) were introduced into the pFP110 plasmid.

Uptake of lactose into *Corynebacterium glutamicum* is also required for producing 2'-FL because wild-type *Corynebacterium glutamicum* is unable to assimilate lactose. Therefore, lactose permease (*lacY*) from *E. coli* K12 was introduced in the pFP110 plasmid. Finally, α-1,2-fucosyltransferase (*α-1,2-ft*) from *Pseudopedobacter saltans* ATCC 51119 was introduced into the pFP110. The heterologous genes are expressed by a polycistronic gene expression cassette controlled by tuf promoter and T7 terminator in the pFP110 plasmid (Appendix B, Figure B.3). Transformation of the pFP110 plasmid into *Corynebacterium glutamicum* ATCC 13032 resulted in the production of 2'-FL from a mixture of lactose and glucose.

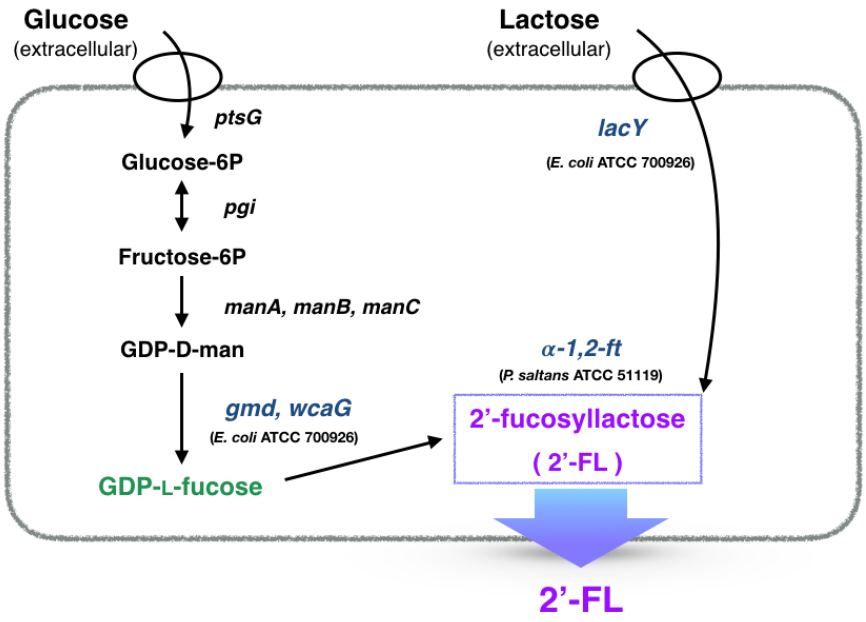

Figure 2. Metabolic Pathway for 2'-Fucosyllactose Biosynthesis in Genetically Modified *Corynebacterium glutamicum* APC199

Where:
ptsG = phosphotransferase system (PTS) glucose-specific enzyme II CB (EIICB) component;

pgi = glucose-6-phosphate isomerase;
manA = mannose-6-phosphate isomerase;
manB = phosphomannomutase;
manC = mannose-1-phosphate guanylyltransferase;
gmd = GDP-D-mannose-4,6-dehydratase;
wcaG = GDP-L-fucose synthase,
lacY = lactose permease;
α-1,2-ft = α -1,2-fucosyltransferase,
Glucose-6P = glucose-6-phosphate;
Fructose-6P = fructose-6-phosphate,
GDP-D-man = GDP-D-mannose;
2'-FL = 2'-fucosyllactose.

As shown in Table 2, the gene encoding α -1,2-fucosyltransferase (*α-1,2-ft*) is originated from a non-pathogenic, non-toxigenic strain of *Pseudopedobacter saltans* ATCC 51119. The genes encoding the three enzymes, GDP-D-mannose-4,6-dehydratase (*gmd*), GDP-L-fucose synthase (*wcaG*), and lactose permease (*lacY*) originated from a non-pathogenic, non-toxigenic strain of *E. coli* ATCC 700926 strain. A detailed description of the production strain construction is included in Appendix B.

Table 2. Introduced Genes in pFP110 plasmid

Gene	Origin	Function	Position in the plasmid
tuf promoter	*Corynebacterium glutamicum* ATCC 13032	Promoter (transcription start)	2182-2381, 200 bp
α-1,2-ft	*Pseudopedobacter saltans* ATCC 51119	α -1,2-fucosyltransferase	2382-3188, 807 bp
gmd	*E. coli* ATCC 700926	GDP-D-mannose-4,6-dehydratase	3220-4341, 1122 bp
wcaG	*E. coli* ATCC 700926	GDP-L-fucose synthase	4367-5332, 966 bp
lacY	*E. coli* ATCC 700926	Lactose permease	5366-6619, 1254 bp
T7 terminator	pET21a plasmid	Transcription termination	6747-6794, 48 bp

GDP=guanosine diphosphate.

Genetic Stability of pFP110 plasmid in *Corynebacterium glutamicum* APC199

In order to verify the absence of non-intentional variations, such as the addition, insertion, and deletion of the sequence of the pFP110 plasmid, the entire sequence of the plasmid DNA was analyzed from multiple-passage cultures of *Corynebacterium glutamicum* APC199. Non-intentional variations were not detected after 32 passages of culture of *Corynebacterium glutamicum* APC199 on non-selective media. A detailed description is included in Appendix C.

Details of Purification Process

In the first step of the purification process, the culture supernatant containing 2'-FL after microfiltration is de-colorized by treatment with activated carbon to remove colorants present in the supernatant (Figure 3). Subsequently, macromolecules are removed by ultrafiltration with a 1.5 kDa molecular weight cut-off (MWCO) membrane. The resulting ultrafiltration permeate is purified through a nanofiltration process. This nanofiltration can remove very small molecules (MWCO 300 Da), such as monosaccharides, disaccharides, amino acids, organic acids, minerals, and salts. Next, an additional activated carbon treatment is performed to remove traces of coloring and organic materials, followed by an ion-exchange chromatography step to remove charged compounds (e.g., peptides, organic acids, and inorganic salts). Potential microbial contaminants during the purification processes are then removed by microfiltration with a 0.25 μm membrane, and the solution is concentrated prior to the crystallization process. Crystallization is induced by adding an anti-solvent (acetic acid or ethanol). Formed 2’-FL crystals are washed with the fresh anti-solvent and dried under vacuum to obtain a high-purity white powder form of 2’-FL. Figure 3 presents the flow diagram of the 2’-FL manufacturing process by APTech.

Tables 3-1 and 3-2 summarize the list of raw materials and processing aids, respectively. All raw materials meet FCC standards or are food grade.

Table 3-1. List of Raw Materials Used in the Fermentation Medium

Fermentation media ingredient	Regulatory Status
Baker’s yeast extract	21 CFR 184.1983
Sodium chloride	21 CFR 182.1(a)
Glucose	21 CFR 184.1857
Lactose*	21 CFR 168.122 (Food standard)
Ammonium sulfate	21 CFR 184.1143
Potassium phosphate monobasic**	No CFR citation for the intended use
Potassium phosphate dibasic	21 CFR 182.6285
Magnesium sulfate heptahydrate	21 CFR 184.1443
Calcium chloride	21 CFR 184.1193
Ferrous sulfate (heptahydrate)	21 CFR 184.1315
Zinc sulfate	21 CFR 182.8997
Copper sulfate	21 CFR 184.1261
Manganese (II) sulfate	21 CFR 184.1461
Ammonium hydroxide	21 CFR 184.1139

*Lactose – It is listed in the Substances Added to Food inventory: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances&sort=Sortterm&order=ASC&showAll=true&type=basic&search=lactose.

**Potassium phosphate, monobasic – SCOG report #32. Sodium phosphate, monobasic is listed at 21 CFR 182.6778. FDA has not objected to the substitution of potassium for sodium in these cases.

https://www.accessdata.fda.gov/scripts/fdcc/?set=SCOGS&sort=Sortsubstance&order=ASC&startrow=1&type=basic&search=phosphate.

It is listed in the Substances Added to Food (formerly EAFUS) inventory: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances&sort=Sortterm&order=ASC&showAll=true&type=basic&search=potassium%20phosphate.

Table 3-2. Processing Aids for Purification of 2'-FL

Materials	Function	Regulatory Status
Activated carbon*	Purification aid	No CFR citation for the intended use
Ultrafiltration membrane	Filtration aid	21 CFR 177.2550
Nanofiltration membrane	Filtration aid	21 CFR 177.2550
Strongly acidic cation exchange resin	Purification aid	21 CFR 173.25
Strongly basic anion exchange resin	Purification aid	21 CFR 173.25
Micro-membrane filter	Filtration aid	21 CFR 177.1520
Acetic acid	Crystallization solvent	21 CFR 184.1005
Ethanol**	Crystallization solvent	No CFR citation for the intended use

*Activated carbon - meets the requirements of the latest version of the Food Chemical Codex (FCC 11th and 12th ed).
It is listed in the Substances Added to Food (formerly EAFUS) inventory: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances&id=ACTIVATEDCARBON

**Ethanol is approved as a processing aid for various purposes but not specifically as a crystallization solvent and is approved as a direct food additive in 21 CFR184.1293.
It is also listed in the Substances Added to Food (formerly EAFUS) inventory: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=FoodSubstances&sort=Sortterm&order=ASC&showAll=true&type=basic&search=ethyl%20alcohol.

Quality Assurance Procedure

APTech is currently building a plant meeting the current Good Manufacturing Practice (cGMP) requirements to manufacture 2'-FL (Appendix D). APTech will observe the principles of Hazard Analysis and Critical Control Point (HACCP)-controlled manufacturing process and cGMP. All processing aids used in the manufacturing process are food grade. All processing aids are suitable for use in food manufacturing, and are compliant with applicable US Federal Regulations, as defined in 21 CFR 173.25 (ion exchange resins), 21 CFR 177.2550, and/or 21 CFR 177.1520.

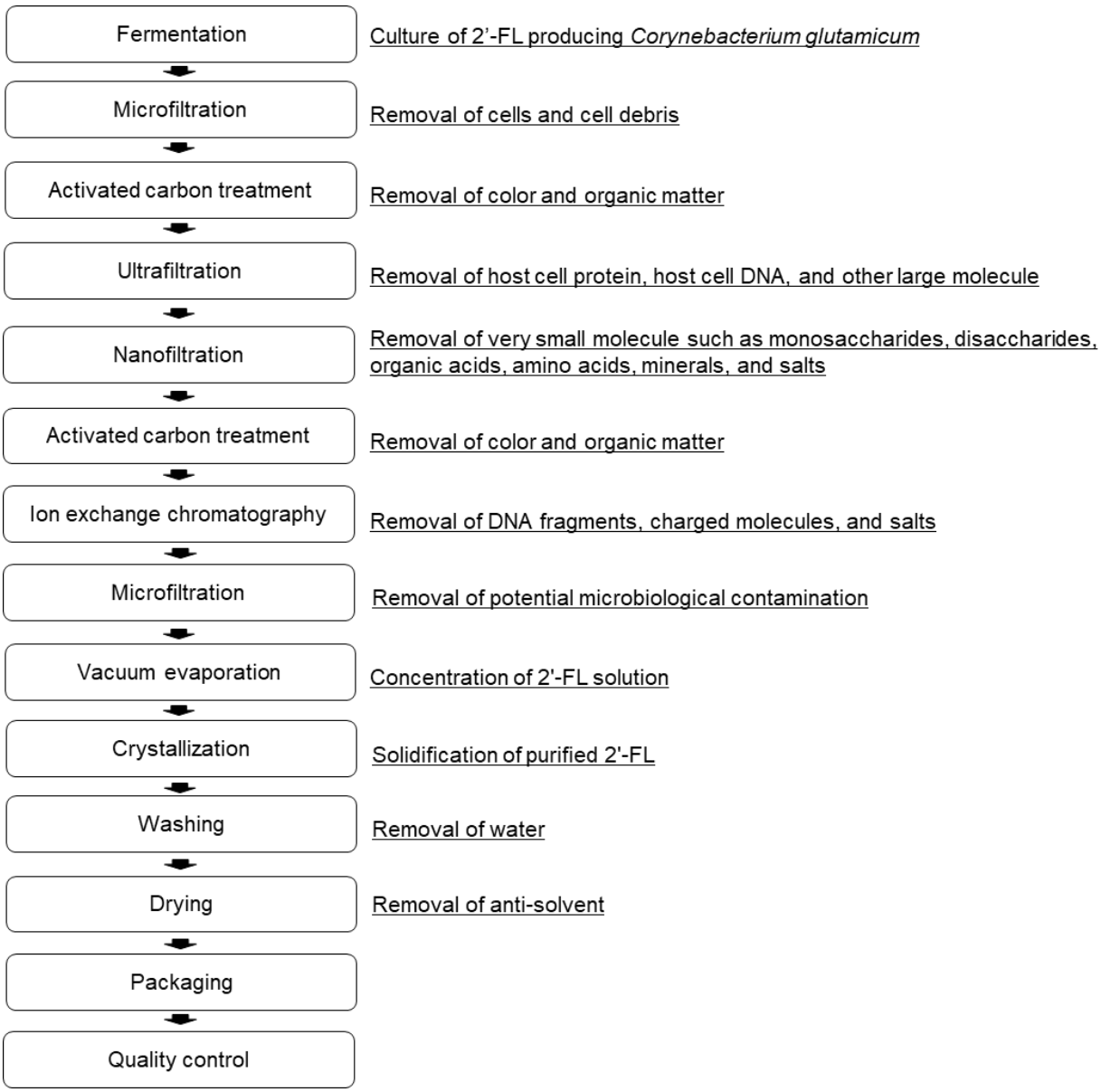

Figure 3. Flow Diagram of the Manufacturing Process

2.C. Identification of the Production Microorganism

Table 4 shows the taxonomic classification of the production microorganism.

Table 4. Taxonomic Classification of *Corynebacterium glutamicum*

Kingdom	*Bacteria*
Phylum	*Actinobacteria*
Class	*Actinobacteria*
Order	*Actinomycetales*
Family	*Corynebacteriaceae*
Genus	*Corynebacterium*
Species	*Corynebacterium glutamicum*
Strain	*Corynebacterium glutamicum* APC199

Corynebacterium glutamicum is a Gram-positive, non-pathogenic bacterium. Various strains of *Corynebacterium glutamicum* have been safety used in the industrial production of an amino acid, such as L-leucine (GRN 523 – FDA, 2014), and carbohydrates, such as D-psicose (GRN 400 - FDA, 2012; GRN 693 - FDA, 2017) and cultured corn starch (GRN 792 -FDA 2019a; GRN 793 - FDA, 2019b).

Corynebacterium glutamicum APC199 was modified from *Corynebacterium glutamicum* ATCC13032 by inserting the pFP110 plasmid and is deposited under Korean Collection for Type Cultures (KCTC) as KCTC 13735BP. A comparative genome analysis of non-pathogenic and non-toxigenic *Corynebacterium glutamicum* APC199 strain (test strain) and *Corynebacterium glutamicum* ATCC13032 was performed to confirm the taxonomic similarity of the two strains (Appendix E). DNA-DNA hybridization (DDH) values have been used by bacterial taxonomists since the 1960s to determine the relatedness between strains and are still the most important criterion in the delineation of the bacterial species. Most recently, the average nucleotide identity (ANI), calculated from pair-wise comparisons of all sequences shared between any two strains, has been proposed as the new metrics for bacterial species classification. Goris et al. (2007) reported 95% similarity of calculated ANI based on whole genome sequencing corresponding to 70% of DDH which is considered to be the gold standard value of species delineation. The comparative ANI value of the test strain and *Corynebacterium glutamicum* ATCC13032 was calculated using whole genome sequence ANI calculating algorithm. The result showed a 99.99% match, indicating a strong similarity between these two strains. Details are presented in Appendix E.

2.D. Specifications and Composition of 2’-FL

Tables 5 and 6 show the specifications and analytical values of 5 non-consecutive batches of APTech’s 2’-FL, respectively. The data demonstrated that the manufacturing process produces a consistent product that is in compliance with the established specifications. All methods of analyses were validated by third party laboratories or by APTech. The product is ≥94% pure on a dry weight basis, as measured by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). Appropriate limits for heavy metals and microbial impurities have been established. The certificates of analysis (COAs) are attached to Appendix F.

Table 7 presents the specifications of APTech’s 2’-FL in comparison with those described in other GRAS notices. Our comparison for specifications has focused on 2’-FL preparations that underwent a series of toxicology studies. As shown in Table 7, the specification and composition of APTech’s 2’-FL are comparable to those presented in other GRAS notices, i.e., GRN 546 (FDA, 2015a - pages 7 and 8; Glycom, produced via chemical synthesis), GRN 571 (FDA, 2015b – stamped page 28; Jennewein Biotechnologie; 2’-FL produced via fermentation with genetically modified *E. coli* BL21), GRN 650 (FDA, 2016a - stamped page 21; Glycom A/S; 2’-FL via fermentation with genetically modified *E. coli* K12), and GRN 735 (FDA, 2018a - pages 22 to 24; Glycosyn, LLC and FrieslandCampina Domo B.V.; 2’-FL via fermentation with genetically modified *E. coli* K12).

The specifications for purified 2’-FL preparations (which provide toxicity study data) ranged from ≥90% (GRNs 571 and 735) to ≥95% (GRN 546). APTech’s specification for 2’-FL is ≥94%, the same as that of GRN 650. It is noteworthy that, as shown in Tables 5 and 7, Enterobacteriaceae and aflatoxin M1 are not part of the specifications for APTech’s 2’-FL, although most other GRAS notices included these two parameters in their specifications. In all specifications, *Cronobacter sakazakii* and *Salmonella* are included as the U.S. food regulations require such microbial tests for infant formulas.

Table 5. Specifications of APTech's 2'-FL

Parameters	Specification	Methods of analysis
Appearance (Color)	White to off white/ivory	USP 34 Rev. <994> or equivalent
Appearance (Form)	Dry powder	
Solubility in water	500 g/L (25°C)	
Appearance in solution	Clear, colorless to slightly yellow	
Water content, %	≤ 9.0	Karl Fischer titration, ASTM E203, or equivalent
Protein content, µg/g	≤ 100	Bradford assay; Bio-rad protein assay #5000006
Total ash, %	≤ 0.5	AOAC 923.03 or equivalent
Arsenic, mg/kg	≤ 0.1	IEC 62321-4 (2014), IEC 62321-5 (2014), ISO 17294:2014, or equivalent
Cadmium, mg/kg	≤ 0.01	
Lead, mg/kg	≤ 0.02	
Mercury, mg/kg	≤ 0.05	
2'-Fucosyllactose, %	≥ 94	Validated HPAEC-PAD
Lactose, %	≤ 5 (Area)	
3-Fucosyllactose, %	≤ 5 (Area)	
Difucosyllactose, %	≤ 5 (Area)	
Fucosyl-Galactose, %	≤ 3 (Area)	
Glucose, %	≤ 3 (Area)	
Galactose, %	≤ 3 (Area)	
Fucose, %	≤ 3 (Area)	
Standard Plate Count, cfu/g	≤ 500	AOAC 990.12 or equivalent
Yeast and Mold, cfu/g	≤ 100	ISO 21527-2 or equivalent
Coliform, cfu/g	≤ 10	AOAC 991.14 or equivalent
Escherichia coli	Absent in 25 g	USP E2022
Cronobacter sakazakii	Absent in 10 g	ISO/TS 22964|IDF/RM 210:2006
Staphylococcus aureus	Absent in 1 g	ISO 6888-1 or equivalent
Salmonella	Absent in 25 g	ISO 6579-1 or equivalent
Endotoxins, EU/g	≤ 100	Ph. Eur. 2.6.14; Endosafe®-PTS™ (Version7.12B, Device4486) cartridge type kit (Charles River)

Abbreviations: USP = The United States Pharmacopeia ; ASTM = The American Society for Testing and Materials ; IEC = International Electrotechnical Commission ; AOAC = Association of Official Analytical Chemists ; ISO = International Organization for Standardization ; HPAEC-PAD = High performance anion exchange chromatography pulsed amperometric detection ; IDF = International Dairy Federation ; cfu = colony forming units ; Ph. Eur = European Pharmacopoeia

Table 6. Analysis of Production Batches of 2’-FL

Parameters	Specification	Batch Number				
		2’-FL-CG-011	2’-FL-CG-012	2’-FL-CG-013	2’-FL-CG-014	2’-FL-CG-015
Manufacturing date		Oct 29, 2018	Dec 10, 2018	Dec 17, 2018	Dec 20, 2018	Jan 02, 2019
Appearance (Color)	White to off white/ivory	Pass	Pass	Pass	Pass	Pass
Appearance (Form)	Dry powder	Pass	Pass	Pass	Pass	Pass
Solubility in water	500 g/L (25°C)	Pass	Pass	Pass	Pass	Pass
Appearance in solution	Clear, colorless to slightly yellow	Pass	Pass	Pass	Pass	Pass
Chemical						
Water content, %	≤ 9.0	1.67	1.74	1.64	2.46	2.70
Protein content µg/g	≤ 100	< 10	< 10	< 10	< 10	< 10
Total ash, %	≤ 0.5	0.17	0.15	0.14	0.03	0.09
Arsenic, mg/kg	≤ 0.1	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Cadmium, mg/kg	≤ 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Lead, mg/kg	≤ 0.02	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Mercury, mg/kg	≤ 0.05	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Carbohydrate content						
2’-Fucosyllactose, %	≥ 94	96.67	95.93	96.24	96.84	97.99
Lactose, %	≤ 5 (Area)	0.10	0.09	0.10	0.11	0.09
3-Fucosyllactose, %	≤ 5 (Area)	ND	ND	ND	ND	ND
Difucosyllactose, %	≤ 5 (Area)	0.24	0.86	0.58	0.02	0.02
Fucosyl-Galactose, %	≤ 3 (Area)	ND	ND	ND	ND	ND
Glucose, %	≤ 3 (Area)	1.13	1.28	1.22	1.00	0.63
Galactose, %	≤ 3 (Area)	0.78	0.78	0.78	0.66	0.43

Fucose, %	≤ 3 (Area)	ND	ND	ND	ND	ND
Microbiology analysis						
Standard Plate Count, cfu/g	≤ 500	0	0	0	0	0
Yeast and Mold, cfu/g	≤ 100	0	0	0	0	0
Coliform, cfu/g	≤ 10	0	0	0	0	0
Escherichia coli, cfu/25 g	ND in 25 g	ND in 25 g	ND in 25 g	ND in 25 g	ND in 25 g	ND in 25 g
Cronobacter sakazakii, cfu/10 g	ND in 10 g	ND in 10 g	ND in 10 g	ND in 10 g	ND in 10 g	ND in 10 g
Staphylococcus aureus, cfu/g	ND in 1 g	ND in 1 g	ND in 1 g	ND in 1 g	ND in 1 g	ND in 1 g
Salmonella, cfu/25 g	ND in 25 g	ND in 25 g	ND in 25 g	ND in 25 g	ND in 25 g	ND in 25 g
Endotoxins, EU/g	≤ 100	< 7.2	< 5.7	< 5	< 5	35.5

ND: Not Detected

Table 7. Comparison of Purified 2'-FL Specifications

Physical and Chemical Parameters	2'-FL Produced by Fermentation				Synthetic 2'-FL
	APTech	Glycosyn and FrieslandCampina (GRN 735)	Glycom (GRN 650)	Jennewein (GRN 571)	Glycom (GRN 546)
Appearance, Form	Dry powder	Homogenous powder	Powder or agglomerates	Spray-dried powder	Powder
Appearance, Color	White to off-white/ivory	White	White to off white	White to ivory-colored	White to off white
Assay, HPAEC or HPLC	≥ 94% (Area, dry wt basis)	≥ 90%	≥ 94.0% (water free)	≥ 90% (area)	≥ 95.0% (water free)
Water, %	≤ 9.0%	≤ 5	≤ 5.0%	≤ 9.0%	≤ 9.0%
Ash, %	≤ 0.5%	≤ 0.2 (sulfated)	≤ 1.5% (sulfated)	≤ 0.5%	≤ 0.2% (Sulphated)
Acetic acid (as free acid and/or sodium acetate)	NS	NS	≤ 1.0%	NS	≤ 0.3%
Residual proteins	≤ 100 µg/g	≤ 0.01%	≤ 0.01%	≤ 100 µg/g	0.1 %
Aluminum, ppm	NS	≤ 4.8	NS	NS	NS
Lead, ppm	≤ 0.02	≤ 0.05	≤ 0.1	≤ 0.02	≤ 0.8
Arsenic, ppm	≤ 0.1	≤ 0.1	NS	≤ 0.2	NS
Cadmium, ppm	≤ 0.01	≤ 0.01	NS	≤ 0.1	NS
Mercury, ppm	≤ 0.05	≤ 0.05	NS	≤ 0.5	NS
Lactose, %	≤ 5 (Area)	≤ 3%	≤ 3%	≤ 5% (Area)	NS
Difucosyllactose, %	≤ 5 (Area)	NS	≤ 1.0	≤ 5% (Area)	NS
3-FL, %	≤ 5 (Area)	NS	NS	≤ 5% (Area)	NS
Fucosyl-galactose, %	≤ 3 (Area)	NS	NS	≤ 3% (Area)	NS
2'-Fucosyl-D-lactulose, %	NS	NS	≤ 1.0	NS	NS
Glucose, %	≤ 3 (Area)	≤ 2%	NS	≤ 3% (Area)	NS
Galactose, %	≤ 3 (Area)	≤ 2%	NS	≤ 3% (Area)	NS
Fucose, %	≤ 3 (Area)	≤ 2%	≤ 1.0	≤ 3% (Area)	NS

Allo-lactose, %	NS	≤ 2%	NS	NS	NS
Total HMOs, %	NS	NS	≥96	NS	NS
Total plate count or aerobic mesophilic total count, CFU/g	≤ 500	≤ 3,000	≤ 500	≤ 10,000	≤ 500
Yeast, CFU/g	≤ 100 (Yeast and Mold)	≤ 10	≤ 10	≤ 100 (Yeast and Mold)	≤ 10
Mold, CFU/g		≤ 10	≤ 10		≤ 10
Salmonella	ND in 25 g	ND in 25 g	ND in 25 g	ND in 100 g	ND in 25 g
Enterobacteriaceae	NS	ND in 10 g	ND in 10 g	ND in 11 g (w/ Coliform)	ND in 10 g
Cronobacter sakazakii	ND in 10 g	ND in 25 g	ND in 10 g	ND in 100 g	ND in 10 g
Listeria monocytogenes	NS	NS	ND in 25 g	NS	ND in 25 g
Bacillus cereus, cfu/g	NS	≤ 100 (presumptive)	≤ 50	NS	≤ 50
Escherichia coli	ND in 25 g	ND in 10 g	NS	NS	NS
Staphylococcus aureus, cfu/g	ND in 1 g	ND in 1 g	NS	NS	NS
Sulphite reducing *clostridia* spores, cfu/g	NS	≤ 30	NS	NS	NS
Clostridium perfringens, cfu/g	NS	ND in 1 g	NS	NS	NS
Residual endotoxins, EU/g	≤ 100	≤ 10,000	NS	≤ 300	≤ 50,000
Aflatoxin M_1, ug/kg	NS	≤ 0.2	NS	≤ 0.025	NS
Residual GMO detection	NS	Negative	NS	Negative	NS

Expanded from GRNs 735. ND=not detected; NS=not specified.

2.E. Chemical Identity and Potential Impurities

Absence of Host Organism, Introduced Antibiotic Resistant Genes, and Enzyme Residues

The microorganism used in the manufacturing process is efficiently removed by the ultrafiltration step. Additionally, during downstream processing, various sequential purification processes are also applied to ensure microbiological purity.

The absence of the microorganism in the ingredient is supported by analysis of residual DNA in batches of the final ingredient by validated quantitative polymerase chain reaction (qPCR) methods. The detection of the genes from the plasmid was used as a proxy for the presence of the host organism. The levels of residual genes [host DNA and four foreign genes (*gmd*, *wcaG*, *α-1,2-ft*, and *lacY*)] were below detection levels. Details are presented in Appendix G.

Microbial Endotoxins

Regulatory threshold levels for food, regarding endotoxin contamination, currently do not exist. Typical ranges of endotoxin load have been reported for cow’s milk (Gehring et al., 2008) and infant formula powder (Townsend et al., 2007). The 2’-FL specification for endotoxin is established to ensure exposures do not exceed the usual levels that are expected for infant formula powder currently on the market. Batch analyses of 2’-FL demonstrate compliance to the endotoxin specification.

Chemical Identity of APTech’s 2’-FL

High-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), mass spectrometric (MS) analysis, and nuclear magnetic resonance (NMR) confirmed that the 2’-FL manufactured by APTech is chemically and structurally identical to those of the reference materials (Carbosynth Ltd. [synthetic 2’-FL] and/or IsoSep [isolated from human milk]). The analysis confirmed the chemical equivalence of APTech’s 2'-FL to 2'-FL references as follows.

1) HPAEC-PAD analysis
   Similar retention times were observed for the main component (APTech’s 2'-FL vs. the reference 2'-FL (7.86 min vs. 7.74 min). The data indicate that APTech’s 2'-FL mimics or is identical to 2'-FL reference (Details are found in Appendix H).

2) Mass spectra
   Identical positive fragmentation patterns were observed comparing the main component of the APTech’s 2'-FL to 2'-FL reference (Carbosynth; synthetic 2’-FL).

3) NMR analysis
   Identity of the product was confirmed by comparison with the ^{1}H and ^{13}C NMR spectra of APTech 2’-FL to reference samples purchased from Carbosynth (synthetic 2’-FL) and IsoSep (2’-FL isolated from human milk).
   Details are presented in Appendix H.

Other Components Present in APTech’s 2'-FL

APTech’s 2'-FL also contains small amounts of other oligosaccharides as identified by HPAEC-PAD analysis: lactose (0.1%), difucosyllactose (0.34%), glucose (1.05%), and galactose (0.69%). All these mono- and disaccharides are components of breast milk and/or infant formula. These concentrations will result in quantitatively insignificant carry-over into the finished infant formula.

2.F. Stability of 2’-FL

APTech has completed a 9-months accelerated storage and 9-months shelf stability study on its 2'-FL. Table 8 summarizes the stability of APTech’s 2’-FL. The 2’-FL samples used in this stability study were in 2 forms: 1) powder form, and 2) 45% in aqueous solution. The samples were analyzed in triplicate for each test point. The 2’-FL powder samples were placed in sterile amber glass bottles with a high-density polyester (HDPE) stopper before being placed into two storage conditions: 1) ambient temperature (25°C/60% relative humidity), and 2) accelerated storage conditions (40°C/75% relative humidity). The liquid samples were prepared by dissolving 2’-FL in purified water to a concentration of 45% (w/v) and placed in sterile amber glass bottles in the same manner as the powder.

At accelerated conditions (40°C at a relative humidity of 75%), 100.5% recovery was reported at week 39 when compared to the baseline value. The data indicated that 2'-FL produced via genetically engineered *Corynebacterium glutamicum* APC199 is as stable as other 2’-FL ingredients prepared by other manufacturing processes.

Table 8. Stability of APTech’s 2'-FL

		Weeks						
		0	1	4	8	13	26	39
% purity								
Powder	25°C/60% RH	96.3	96.8	96.8	96.8	96.7	97.3	97.1
	40°C/75% RH	96.3	96.8	96.9	96.7	96.6	97.0	96.8
45% Solution	25°C/60% RH	96.8	96.8	96.7	96.3	95.8	95.6	94.5
% recovery compared to the baseline								
Powder	25°C/60% RH	100.0	100.6	100.5	100.5	100.4	101.0	100.9
	40°C/75% RH	100.0	100.6	100.6	100.4	100.3	100.8	100.5
45% Solution	25°C/60% RH	100.0	100.0	99.9	99.5	98.9	98.7	97.6

RH=relative humidity

As shown in Table 9, stability tests were conducted by various companies. Because the purity and composition of APTech’s 2’-FL preparation are similar to those described in previous GRAS notices, the information and data in other GRAS notices are pertinent to the expectation of stability of APTech’s 2’-FL.

DuPont Nutrition (GRN 749, pages 17 to 19 - FDA, 2018b) reported that its 2’-FL, manufactured via genetically engineered *E. coli* K12, was shelf stable for up to 26 weeks at 40°C and 75% relative humidity. Compared to the baseline, an average of 99.6% recovery was reported at 26 weeks in accelerated conditions.

Glycosyn and FrieslandCampina Domo reported the stability of 98% (as compared to the baseline) after 6 months under accelerated and room temperature storage conditions for its 2’-FL manufactured via genetically engineered *E. coli* K12 (GRN 735, pages 27 to 29 - FDA, 2018a).

In GRN 650 (stamped pages 26 to 29 - FDA, 2016a), Glycom indicated that its fermentation-produced 2'-FL, manufactured via genetically engineered *E. coli* K12, was stable. At accelerated conditions (80°C or 60°C and ambient humidity), 99.8 to 101.5% of the baseline value were reported after 3 months of storage.

In GRN 571 (stamped pages 29 to 30 - FDA, 2015b), Jennewein indicated that its 2'-FL, produced by genetically engineered *E. coli* BL21, had a shelf-life of at least 2 years. At week 104, an average of 106.6% of the baseline values was recovered when stored at 25°C and a relative humidity of 60%. At an accelerated condition (40°C and a relative humidity of 75%), an average of 103.5% of the baseline value was recovered at week 26.

In GRN 546 (pages 11 to 13 - FDA, 2015a), Glycom reported the bulk shelf-stability of 36 months with a 97.0% recovery for its 2'-FL, prepared via chemical synthesis (≥95% purity), when stored at 25°C and a relative humidity of 60%. Under accelerated conditions (at 40°C and a relative humidity of 75%), they reported a shelf-stability of 6 months with a 98.2% recovery.

In GRN 852 (page 10, Annex IV – FDA, 2019c), BASF reported a 101.0% of the baseline value after 6 months of storage under accelerated conditions (at 40°C and a relative humidity of 75%).

Stability in Infant formula and conventional foods

GRN 546 (pages 13 to 17 - FDA, 2015a) reported that chemically synthesized 2'-FL was stable in conventional foods and infant formula. No significant loss of 2’-FL was observed under any of the storage conditions for up to 540 days (Table 9). Compared to the baseline, an average of 103.2% recovery was reported at 540 days after storage at 37°C. In addition, 2'-FL was stable in other foods, including yoghurt, citrus fruit drinks, and ready-to-drink chocolate-flavored milk when prepared and stored under the recommended conditions (GRN 546, pages 15 to 17 - FDA, 2015a).

Overall, the stability data reported for 2'-FL in previous GRNs (FDA, 2015a; 2015b; 2016a; 2018a; 2018b) support that all purified 2'-FL preparations, regardless of methods of manufacture, are shelf stable and well-suited for the intended food uses. Because APTech’s 2’-FL has a purity of ≥ 94%, the shelf stability is expected to be similar to those of other 2’-FL preparations.

Table 9. Stability of Other Sources of 2’-FL in Bulk Powder and Powdered Infant Formula at Room Temperature

GRN	**Food matrix**	**Test Conditions**	
		Accelerated, 40°C 75% RH	**Shelf-stability, 25°C 60% RH**
749	Bulk powder	99.6% at week 26	
735	Bulk powder	97.8% at 6 mo	98.4% at 6 mo
650	Bulk powder	**Accelerated, 60°C and ambient humidity**	
		101.5% at 3 mo	
		Accelerated, 80°C and ambient humidity	
		99.8% at 3 mo	
571	Bulk powder	103.5% at week 26	106.6% at week 104

546	Bulk powder	98.2% at 6 mo (40°C, 75% RH)	97.0% at 36 mo; 99.9% at 24 mo (25°C, 60% RH)
	Infant formula	**37°C**	**4°C, 20°C, and 30°C**
		103.2% at day 540	98.3 - 105.8% at day 540

All the recovery values are in comparison with those at initial points.

2.G. Intended Technical Effects

2’-FL will be used as an ingredient in conventional foods as well as non-exempt infant formulas (whey-, milk-, and/or soy-based) for term infants and formulas intended for toddlers and children aged 12 - 36 months.

PART 3. EXPOSURE ESTIMATES

3.A. Estimated Dietary Intakes (EDIs) of 2’-FL Under the Intended Use

Because 2’-FL will be added to the same food categories at the same use levels described in GRN 735, the EDIs are expected to be the same as or similar to those reported in GRN 735. 2’-FL is intended for use as a food ingredient in term infant formulas, formulas for toddlers and children aged 12-36 months, and selected conventional foods at the levels listed in Table 1.

Based on the food consumption data reported in a recent National Health and Nutrition Examination Survey (NHANES; 2013-2014) dataset compiled by the U.S. Department of Health and Human Services, National Center for Health Statistics, and the Nutrition Coordinating Center, the EDIs of 2’-FL were calculated from the food code list and the survey database of diet recalls.

EDIs of Infant Formula

Table 10 presents the data on infant formula intakes by age, which range from 1,077 to 1,219 g/person/day. On a body weight basis, these intakes correspond to 118 to 226 g/kg body weight (bw)/day.

EDIs of 2’-FL from the Proposed Use in Infant Formulas Only

The estimates for the daily intake of 2’-FL from its use in only term-infant formulas are summarized in Table 11. From the use of 2’-FL in infant formulas only (a maximum level of 2.4 g/L of ready-to-use or reconstituted formula prepared from powder), the estimated mean and 90th percentile intakes of 2’-FL were determined to be at 1.87 and 2.78 g/person/day, respectively, in all-user infants aged 0 to 11.9 months. On a body weight basis, these intakes were estimated to be 258.7 and 431.3 mg/kg bw/day, respectively. The all-user mean and 90th percentile intakes of 2’-FL were estimated to be the highest in infants aged 3 to 5.9 months at 2.04 and 2.93 g/person/day, respectively (Table 11). On a body weight basis, the greatest intake would occur in infants aged 0 - 2.9 months at 347.8 and 541.9 mg/kg bw/day, respectively.

Table 10. EDIs of Infant Formula

Population Group	All-Person Intake		All-Users Intake			
	Mean	90th Pctl	% Users	n	Mean	90th Pctl
g/person/day						
0-2.9 mo	509	1095	66.5	140	766	1212
3-5.9 mo	609	1128	71.8	151	849	1219
6-8.9 mo	629	1069	81.2	162	775	1077
9-11.9 mo	495	1012	68.6	115	721	1156
0-11.9 mo	563	1096	72.2	568	780	1157
g/kg bw/day						
0-2.9 mo	96.3	204.4	66.5	140	144.9	225.8
3-5.9 mo	85.6	170.4	71.8	151	119.2	175.5
6-8.9 mo	74.0	133.4	81.2	162	91.1	140.8
9-11.9 mo	52.8	76.6	68.6	115	76.6	118.3
0-11.9 mo	77.9	168.3	72.2	568	107.8	179.7

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; bw = body weight; mo = months; pctl = percentile.

Table 11. EDIs of 2’-FL from the Proposed Use in Infant Formula Only

Population Group	All-Person Intake		All-Users Intake			
	Mean	90th Pctl	% Users	n	Mean	90th Pctl
g/person/day						
0-2.9 mo	1.22	2.63	66.5	140	1.84	2.91
3-5.9 mo	1.46	2.71	71.8	151	2.04	2.93
6-8.9 mo	1.51	2.57	81.2	162	1.86	2.58
9-11.9 mo	1.18	2.43	68.6	115	1.73	2.77
0-11.9 mo	1.35	2.63	72.2	568	1.87	2.78
mg/kg bw/day						
0-2.9 mo	231.1	490.6	66.5	140	347.8	541.9
3-5.9 mo	205.4	409.0	71.8	151	286.1	421.2
6-8.9 mo	177.6	320.2	81.2	162	218.6	337.9
9-11.9 mo	126.7	183.8	68.6	115	183.8	283.9
0-11.9 mo	187.0	403.9	72.2	568	258.7	431.3

Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; bw = body weight; mo = months; pctl = percentile.
Intended use of 2’-FL in infant formula=2.4 g/L.

Infants and All-Age Groups: EDIs of 2’-FL from the Combined Use in Infant Formula and Other Foods and Beverages

As presented in GRN 735 (pages 31 to 33), the mean and 90th percentile EDIs of 2'-FL in all-users of all ages were determined to be 1.70 and 3.54 g/person/day, respectively (Table 12). The mean and 90th percentile intakes in all-user infants were estimated at 1.91 (0 to 5.9 months old infants) to 2.28 (6.0 to 11.9 months) and 3.00 (0 to 5.9 months) to 3.86 g/person/day (6.0 to 11.9 months), respectively. The highest intake was observed to occur in male teenagers with the highest 90th percentile intake at 4.29 g/person/day.

On a body weight basis, the mean and 90th percentile EDIs were 36 and 80 mg/kg bw/day, respectively, in all-users of all ages (Table 13). Of all-users, infants aged 0 to 5.9 months were estimated to have the highest mean and 90th percentile EDIs of 315 and 532 mg/kg bw/day, respectively.

Table 12. Summary of the EDI of 2’-FL from Proposed Combined Use in Infant Formula and Other Foods and Beverages, g/person/day

Population Group	Age Group	All-person (or per capita) Intake (g/d)		All-users Intake (or consumers only, g/d)			
		Mean	90th Pctl	%	n	Mean	90th Pctl
Infants	0-5.9 mo	1.10	2.75	57.5	107	1.91	3.00
	6-11.9 mo	2.14	3.86	94.1	160	2.28	3.86
Toddlers	12-35 mo	1.83	2.97	100.0	348	1.83	2.97
Children	3-11 y	1.96	3.53	99.7	1,277	1.97	3.53
Female teenagers	12-19 y	1.47	2.95	94.7	544	1.55	2.95
Male teenagers	12-19 y	1.85	4.16	92.5	526	2.00	4.29
Women of child-bearing age	16-45 y	1.22	2.82	89.9	1,219	1.36	2.87
Female adults	20+ y	1.32	2.96	91.9	2,169	1.44	3.05
Male adults	20+ y	1.59	3.81	86.8	1,842	1.84	3.97
Elderly	65+ y	1.76	3.74	92.8	939	1.90	3.91
Total population	All ages	1.55	3.41	91.2	6,973	1.70	3.54

Adopted from GRN 735, page 32. Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; mo = months; y = years.

Table 13. Summary of the EDIs of 2’-FL from Proposed Combined Use in Infant Formula and Other Foods and Beverages, mg/kg bw/day

Population Group	Age Group	All-person Intake (mg/kg bw/d)		All-users Intake (mg/kg bw/d)			
		Mean	90th Pctl	%	n	Mean	90th Pctl
Infants	0-5.9 mo	181	477	57.5	107	315	532
	6-11.9 mo	244	441	94.1	160	259	447
Toddlers	12-35 mo	148	243	100.0	346	148	243
Children	3-11 y	75	147	99.7	1,268	76	147
Female teenagers	12-19 y	24	52	94.7	536	26	52
Male teenagers	12-19 y	29	67	92.5	524	31	67
Women of child-bearing age	16-45 y	18	42	89.9	1,209	20	43
Female adults	20+ y	19	42	91.9	2,156	20	43
Male adults	20+ y	19	46	86.7	1,833	22	48
Elderly	65+ y	24	53	92.6	928	26	54
Total population	All ages	32	76	91.1	6,930	36	80

Adopted from GRN 735, pages 32-33. Based on the 2013-2014 National Health and Nutrition Examination Survey (NHANES) dataset; Pctl = percentile; mo = months; y = years.

3.B. Food Sources of 2’-FL

The primary source of 2’-FL in the human diet is from human milk. An average concentration of more than 400 human milk samples from women living in 10 different countries was 2.38 g/L (Castanys-Munoz et al., 2013). The mean 2’-FL concentrations of human milk collected from various cohorts range from 0.22 to 8.4 g/L, depending on the genotype of the mother and stage of lactation (Asakuma et al., 2008, 2011; Austin et al., 2016; Balogh et al., 2015; Bao et al., 2013; Castanys-Munoz et al., 2013; Chaturvedi et al., 1997, 2001a; Coppa et al., 1999, 2011; Donovan and Comstock, 2016; Erney et al., 2000, 2001; Gabrielli et al., 2011; Galeotti et al., 2012, 2014; Goehring et al., 2014; Grollman and Ginsburg, 1967; Hong et al., 2014; Kunz et al., 1999; Leo et al., 2009, 2010; Marx et al., 2014; McGuire et al., 2017; Morrow et al., 2004; Musumeci et al., 2006; Nahkla et al., 1999; Smilowitz et al., 2013; Sumiyoshi et al., 2003; Thurl et al., 1996, 2010). Individual studies are summarized in Appendix I.

3.C. EDIs of 2’-FL from Diet and Other Sources

2’-FL level in each food is not listed in the USDA food composition tables or the National Health and Nutrition Examination Survey (NHANES) databases. In addition, no literature has reported 2’-FL concentrations in foods other than human milk. Thus, the EDIs from the diet were not estimated.

However, GRN 650 (FDA, 2016a -stamped page 33) described that a 6.5-kg infant drinking 1 L of milk per day would be expected to consume 170 to 660 mg/kg bw/day of 2’-FL (based on the mean levels of 2’-FL present in mature human milk) and that the intake of 2’-FL from mature breast milk may be up to 1,150 mg/kg bw/day among infants from secretor mothers.

Despite the fact that there is no New Dietary Ingredient (NDI) status with US FDA for 2’-FL, 2’-FL is currently marketed in the U.S. as a dietary supplement with a recommended daily dose of 400 mg. Thus, the contribution to EDI from the use in dietary supplements is expected to be not significant.

3.D. Total EDIs of 2’-FL from Diet and Under the Intended Use

As mentioned in 3.C., 2’-FL level in each food is not listed in the USDA food composition tables or the National Health and Nutrition Examination Survey (NHANES) databases. Thus, the EDIs from the diet were not estimated. In addition, no literature has reported 2’-FL concentrations in foods other than human milk. The EDIs under the intended use are presented in Part 3.A.

3.E. EDIs of Other Substances Under the Intended Use

Impurities of APTech’s 2’FL ingredient may include lactose (0.1%), difucosyllactose (0.34%), glucose (1.05%), and galactose (0.69%). However, these concentrations may result in quantitatively insignificant carry-over into the finished infant formula.

Summary of Consumption Data

Infants: EDIs of 2’-FL from Infant Formula Use Only

From the use of 2’-FL in only infant formula (a maximum level of 2.4 g/L of ready-to-use or reconstituted formula), the estimated mean and 90th percentile intakes of 2’-FL were determined to be at 1.87 and 2.78 g/person/day, respectively, in all-user infants aged 0 to 11.9 months old. On a body weight basis, these intakes were determined to be 258.7 and 431.3 mg/kg bw/day, respectively. The all-user estimated mean and 90th percentile intakes of 2’-FL were greatest in infants aged 3 to 5.9 months old at 2.04 and 2.93 g/person/day, respectively. On a body weight basis, the greatest intake was

observed to occur in infants aged 0 - 2.9 months at 347.8 and 541.9 mg/kg bw/day, respectively.

Infants and All-Age Groups - EDIs of 2’-FL from the Use of Infant Formula and Other Foods

The mean and 90th percentile EDIs of 2'-FL in all-users of all ages were determined to be 1.70 and 3.54 g/person/day, respectively. The mean and 90th percentile intakes in all-user infants were estimated at 1.91 (0 to 5.9 months old infants) to 2.28 (6.0 to 11.9 months) and 3.00 (0 to 5.9 months) to 3.86 g/person/day (6.0 to 11.9 months), respectively. The highest intake was observed to occur in male teenagers with the highest 90th percentile intake at 4.29 g/person/day. On a body weight basis, the mean and 90th percentile EDIs were 36 and 80 mg/kg bw/day, respectively, in all-users of all ages. Of all-users, infants aged 0 to 5.9 months were estimated to have the highest mean and 90th percentile EDIs of 315 and 532 mg/kg bw/day, respectively.

The proposed use levels are similar to those described in another 2’-FL GRAS notice. The EDI assessments are based on the assumption that APTech’s 2’-FL will replace currently marketed 2’-FL. Thus, cumulative exposures are not expected to change.

The 2’-FL level in each food is not listed in the USDA food composition tables or the National Health and Nutrition Examination Survey (NHANES) databases. In addition, no literature has reported 2’-FL concentrations in foods other than human milk. Thus, the EDIs from the diet were not estimated. 2’-FL is currently marketed as a dietary supplement with a recommended daily dose of 400 mg. The contribution to EDI from the use in dietary supplements is expected to be not significant.

PART 4. SELF LIMITING LEVELS OF USE

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

PART 5. HISTORY OF CONSUMPTION

The statutory basis for the conclusion of GRAS status of 2’-FL in this document is not based on common use in food before 1958.

PART 6. NARRATIVE

6.A. Current Regulatory Status

USA

Various sources of human milk oligosaccharides (HMOs) have been evaluated by the FDA over the past 5 years for incorporation of HMO products in infant formulas for consumption by term infants. Relevant U.S. GRAS notifications include 2’-FL (GRN 546, FDA, 2015a; GRN 571, FDA, 2015b; GRN 650, FDA, 2016a; GRN 735, FDA, 2018a; GRN 749, FDA, 2018b; GRN 852, FDA, 2019c), a mixture of 2’-FL and difucosyllactose (DFL; GRN 815, FDA, 2019d), lacto-*N*-neotetraose (LNnT; GRN 547, FDA, 2015c; GRN 659, FDA, 2016b) and 3’-sialyllactose (GRN 766, FDA, 2018c). FDA had no questions on the use levels of these HMOs similar to those found in human milks. Table 14 summarizes the regulatory status of 2’-FL in the USA.

Table 14. Regulatory Status of 2’-FL in USA

GRN	Substance	Intended Food Uses	Company
546	2’-FL manufactured using chemical synthesis (≥ 95% purity)	Formulas for term infants and toddlers (2.4 g/L); selected foods and beverages (0.084 - 2.4 g/serving)	Glycom A/S (FDA, 2015a)
571	2’-FL manufactured using a GM *E. coli* BL21 (≥ 90% purity)	Formulas for term infants and toddlers (2 g/L)	Jennewein Biotechnologie GmbH (FDA, 2015b)
650	2’-FL manufactured using a GM *E. coli* K12 (≥ 94% purity)	Formulas for term infants and toddlers (2.4 g/L); selected foods and beverages (0.084 - 2.04 g/serving)	Glycom A/S (FDA, 2016a)
735	2’-FL manufactured using a GM *E. coli* K12 (≥ 90% purity)	Formulas for term infants and toddlers (2.4 g/L); infant and toddler foods (0.24 - 1.2 g/serving); selected foods and beverages (0.28 - 1.2 g/serving)	Glycosyn, LLC and FrieslandCampina Domo (FDA, 2018a)
749	2’-FL manufactured using a GM *E. coli* K12 (≥ 82% purity)	Formulas for term infants and toddlers (2.4 g/L); other baby foods for infants and young children (0.84 - 2.04 g/serving or 12 g/kg); other drinks for young children (0.14 g/serving or 1.2 g/kg)	DuPont Nutrition (FDA, 2018b)

852	2’-FL manufactured using a GM *E. coli* K12 (≥ 90% purity)	Non-exempt formulas for term infants (2.4 g/L); formulas for toddlers (2.4 g/L); baby foods (0.24 - 1.2 g/serving): selected foods and beverages (0.28 - 1.2 g/serving)	BASF (FDA, 2019c)
815	A mixture of 2’-FL and DFL (approximate ratio = 8:1)	Formulas for term infants (1.6 g/L); formulas for toddlers and drinks for young children (1.2 g/L); other foods for infants and young children (10 g/kg); other selected foods and beverages (2 - 40 g/kg)	Glycom (FDA, 2019d)

GM=genetically modified; The numbers in parenthesis indicate the maximum use levels.

European Union (EU)

In the EU, 2’-FL is authorized as a novel ingredient (EU, 2018). The European Food Safety Authority (EFSA) panel concluded that 2'-FL is safe for infants, toddlers, and adults:

1) Infants, when added to infant and follow-on formula, in combination with another oligosaccharide, LNnT, at concentrations up to 1.2 g/L of 2'-FL and up to 0.6 g/L of LNnT, at a ratio of 2:1 in the reconstituted formula.

2) Young children (older than one year of age) when added to follow-on and young-child formula at concentrations up to 1.2 g/L of 2'-FL alone or in combination with LNnT, at concentrations up to 0.6 g/L, at a ratio of 2:1.

3) Adults when added to dairy and milk products, dairy analogs, cereal bars, table top sweeteners, dietary foods for weight control diets, beverages, and food supplements at concentrations of 1.2 g/L for beverage products, 1.2-2.4 g/ serving for food products, and 3.0 g/day for food supplements (EFSA, 2015).

Recently, EFSA (2019a) approved a mixture of 2’-FL (≥75%) and difucosyllactose (DFL ≥5%) as a novel food ingredient for infant formulas (1.6 g/L, ready-to use or reconstituted as instructed by the manufacturer) and food supplement applications (up to 2 g/L for beverages and up to 4 wt% for products other than beverages).

6.B. APTech’s 2’-FL is Structurally Identical to that Present in Human Milk

As presented in Part 2.E. (pages 24 - 25) of this notice, APTech’s 2’-FL is chemically and structurally identical to the 2’-FL which is found in human milk.

6.C. Review of Safety Data

To identify other data and information relevant to the safety of infant formula and food uses of 2′-FL, a comprehensive search of the published scientific literature was conducted. Published studies identified during the literature search consisted of studies relating to the metabolic fate and safety of 2’-FL. Most of the studies that form the basis of this safety assessment have been reviewed in GRN 749 (pages 30 to 39 - FDA, 2018b), GRN 735 (pages 50 to 65 - FDA, 2018a), GRN 650 (stamped pages 34 to 40), GRN 571 (stamped pages 39 to 53 - FDA, 2015b), and GRN 546 (pages 29 to 35 – FDA, 2015a).

As described in Part 2.E. of this notice, the 2’-FL in this GRAS determination is structurally identical to that present in human milk and to that evaluated in a previous GRAS notice (synthetic; GRN 546). In addition, the 2’-FL in this GRAS determination has similar specifications compared to those discussed in previous GRAS notices (Table 7). It is recognized that the information and data in published papers are pertinent to the evaluation of the safety of the APTech’s 2’-FL in this GRAS determination. Therefore, this notice incorporates, by reference, the safety and metabolic studies discussed in the previous GRAS notices, and will not discuss previously reviewed references in detail. Additionally, this notice discusses studies that have been published since FDA’s reviews in 2017 - 2019, in particular the papers published between October 2017 and December 2019.

6.C.1. Absorption, Distribution, Metabolism, and Elimination (ADME)

It is generally accepted that most of the HMOs, including 2’-FL, resist digestion in the stomach and the small intestine and reach the large intestine intact. In the colon, they are either fermented by the intestinal microflora or excreted unchanged in the feces (Brand-Miller et al., 1998; Gnoth et al. 2000; Newburg, 2000). From a breath hydrogen test, Brand-Miller et al. (1998) estimated that, on average, all of the load of purified HMO isolated from their mothers’ milk (113% ± 18%) reached the large intestine and was fermented by infants aged 3 to 8 months. An *in vitro* study by Gnoth et al. (2000) demonstrated that less than 5% of the HMO are digested in a simulated intestinal tract condition. Thus, the majority of 2’-FL enters the colon intact and is subjected to partial fermentation by the indigenous microbiota populations therein (Brand-Miller et al., 1998).

Marriage et al. (2015) reported that the relative absorption of 2’-FL in the plasma is in the range of 0.05 and 0.07% for newborn infants receiving formula supplemented with 0.2 and 1.0 g 2’-FL/L, respectively.

Studies showed that HMOs already appear in maternal urine and blood during pregnancy and as early as the first trimester (Jantscher-Krenn et al., 2019; Wise et al., 2018). Wise et al. (2018) reported that HMOs also appear in amniotic fluid: several HMOs, including 2'-FL, 3-FL, DFL, and 6'- sialyllactose, were present in different relative abundancies in all three samples (urine, milk, and amniotic fluid). The data indicate that HMOs appear in amniotic fluid and that the fetus is already exposed to HMOs *in utero.*

6.C.2. Mutagenicity and Genotoxicity Studies

6.C.2.1. Published Mutagenicity and Genotoxicity Studies

In published studies summarized in Table 15-1, 2’-FL preparations (synthetic and made via fermentation with genetically modified *E. coli* K12) were found to be not mutagenic or genotoxic in the bacterial reverse mutation test, the micronucleus test in cultured human lymphocytes, or in L5178Y tk+/- mouse lymphoma cells (Coulet et al., 2014; van Berlo et al., 2018). In a study by Phipps et al. (2018), a mixture of 2’-FL and difucosyllactose (DFL) (approximately 8:1 ratio) also did not show any mutagenicity and genotoxicity in the bacterial reverse mutation test or in human peripheral blood lymphocytes (Table 15-1).

Table 15-1. Summary of Published Mutagenicity or Genotoxicity Studies

Test System	Concentration/Dose	Result	Reference
Recent Studies of Other Sources of 2’-FL			
S. typhimurium TA98, TA100, TA1535, TA1537, and *E. coli* WP2*uvr*A (pKM101)	5, 15, 50, 150, 500, 1,500, or 5,000 µg/plate a mixture of 2’-FL (82.5% w/w) and DFL (9.7 % w/w) ± S9 (source-Glycom)	Not mutagenic and not genotoxic	Phipps et al., 2018
Human peripheral blood lymphocytes	500, 1,000, or 2,000 µg/mL 2’-FL/DFL mixture; short term exposure ± S9; long term exposure -S9 (source-Glycom)		
S. typhimurium TA98, TA100, TA1535, TA1537, and *E. coli* WP2*uvr*A; ± S9	62, 185, 556, 1,667, or 5,000 µg/plate 2’-FL (Source - Glycosyn and FrieslandCampina, purity 94%)	Not mutagenic	van Berlo et al., 2018 (the same study was reported in GRN 735, page 56 - FDA, 2018a)
In vitro micronucleus test in cultured human lymphocytes ± S9	500, 1,000, or 2,000 µg/mL 2’-FL (Source - Glycosyn and Friesland Campina, purity 94%)	Not genotoxic	

S. typhimurium TA98, TA100, TA102, TA1535, TA1537 ± S9	Plate incorporation method, 52, 164, 512, 1,600, or 5,000 µg 2'-FL/plate; pre-incubation method, 492, 878, 1,568, 2,800, or 5,000 ug/plate (source-Glycom, synthetic; purity >99%, dw basis)	Not mutagenic and not genotoxic	Coulet et al., 2014 (the study was reported in GRN 546, pages 32 to 33 FDA, 2015a)
L5178Y tk+/- mouse lymphoma cells ± S9	Long term treatment, 1.7, 5.4, 17, 52, 164, 512, 1,600 or 5,000 µg/mL -S9; Short term treatment, 492, 878, 1,568, 2,800 or 5,000 µg/mL ± S9 (source - Glycom, synthetic; purity >99%, dw basis)		

6.C.2.2. Unpublished Mutagenicity and Genotoxicity Studies

As shown in Table 15-2, 2'-FL had no mutagenicity and genotoxicity in the bacterial reverse mutation test (Case and Yoon, 2020 [Appendix J]; Verspeek-Rip, 2015), *in vitro* chromosome aberration test with Chinese Hamster Lung (CHL/IU) cells (Case and Yoon, 2020), *in vivo* micronucleus test in ICR mice (Case and Yoon, 2020), *in vitro* micronucleus test with cultured human blood peripheral lymphocytes (Verbaan, 2015), and/or in the *in vivo* micronucleus test in bone marrow cells of the (Crl:CD(SD)) rats (GRN 571 - FDA, 2015b).

Results from unpublished studies of APTech's 2'-FL and other 2'-FL preparations (manufactured via genetically modified *E. coli* K12 or BL21 strains) confirmed the findings from published studies. The unpublished status of these corroborative studies has no impact on the overall conclusion of this GRAS determination if qualified experts do not have access to such data.

Table 15-2. Summary of Unpublished Mutagenicity or Genotoxicity Studies of 2'-FL

Test System	2'-FL Conc./Dose for test groups	Result	Reference
Bacterial reverse mutation test: *S. typhimurium* (TA98, TA100, TA1535, and TA1537) and *E. coli* WP2*uvrA* (pKM101)	Main study, 313, 625, 1,250, 2,500, and 5,000 µg/plate (purity, ≥94%; source - APTech)	Not mutagenic	Case and Yoon, 2020 (Appendix J)
In vitro chromosome aberration test: Chinese Hamster Lung (CHL/IU) cells	Main study, 1,250, 2,500, and 5,000 µg/mL (purity, ≥94%; source - APTech)	Not clastogenic	Case and Yoon, 2020 (Appendix J)
In vivo micronucleus test: ICR mice	Main study, 2,500, 5,000, and 7,500 mg/kg bw (purity, ≥94%; source -APTech)	Not genotoxic	Case and Yoon, 2020 (Appendix J)
S. typhimurium TA98, TA100, TA1535, TA1537, and *E. coli* WP2uvrA ± S9	Plate incorporation method, 52, 164, 512, 1,600, and 5,000 µg 2'-FL/plate; pre-incubation method 492, 878, 1,568, 2,800 and 5,000 ug/plate (purity, 97.6%; source - Glycom)	Not mutagenic	Verspeek-Rip, 2015 (GRN 650, stamped page 42; FDA, 2016a)
In vitro micronucleus test with cultured human blood peripheral lymphocytes ± S9	Short term treatment, 512, 1,600, and 2,000 µg/mL ± S9; Long term treatment, 512, 1,600, and 2,000 µg/mL -S9 (purity, 97.6%; source - Glycom)	Not clastogenic and not aneugenic	Verbaan, 2015 (GRN 650, stamped page 42-43; FDA, 2016a)
S. typhimurium TA98, TA100, TA102, TA1535, and TA1537 ± S9	Plate incorporation method, 31.6, 100, 316, 1,000, 3,160 and 5,000 µg/plate (purity, 92.4%; source - Jennewein)	Not mutagenic	GRN 571 (stamped pages 40 to 41; Appendices M1 (p 398) and M2 (p 413 - FDA, 2015b)
In vivo micronucleus test: (Crl:CD(SD)) rats	500, 1,000, and 2,000 mg/kg bw/d (purity, 96.0%; source - Jennewein)	Not genotoxic	

6.C.3. Animal Toxicity Studies

Because the 2'-FL in this GRAS determination has similar specifications compared to those described in previous GRAS notices (Table 7) and APTech's 2'-FL is structurally identical to that evaluated in a published toxicity study (synthetic 2'-FL described in Coulet et al., 2014 and in GRN 546-FDA, 2015a), the information and data in those GRAS notices are pertinent to the evaluation of safety of the APTech's 2'-FL in this GRAS determination.

6.C.3.1. Published Animal Toxicity Studies

Table 16-1 summarizes published toxicity studies of 2'-FL and the mixture of 2'-FL and DFL. As shown in Tables 16-1, purified 2'-FL preparations showed similar toxicology profiles (synthetic vs. fermentation via genetically modified *E. coli* K12). From a subchronic oral toxicity study, the NOAEL for synthetic 2'-FL was placed at 5,000 mg/kg bw/day in both male and female rats (Coulet et al., 2014). The NOAELs for the preparation manufactured via genetically engineered *E. coli* K12 were determined to be the highest levels tested, i.e., 7,250 mg/kg bw/day for male rats and 7,760 mg/kg bw/day for female rats (van Berlo et al., 2018). A subchronic oral toxicity study of the 8:1 mixture of 2'-FL and DFL found the NOAEL as 5,000 mg/kg bw/day, the highest level tested (Phipps et al., 2018). The NOAEL of 2'-FL is at least 5,000 mg/kg bw/day in rats, regardless of methods of manufacture.

Table 16-1. Summary of Published Toxicity Studies of 2'-FL

Animal	2'-FL Dose	Duration	Results	Reference
Published Studies				
Wistar Crl:WI (Han) rats	0, 2,000, 5,000, or 7,500 mg 2'-FL/kg bw/d (99% purity on a dry weight basis; source - Glycom, synthetic 2'-FL), or 7,500 mg/kg bw/d FOS (reference)	14 d (PND 7 to 20)	The suitable dose of 2'-FL should be lower than 7,500 mg/kg bw/d for male and female rats	Coulet et al., 2014 (this study was described in GRN 546, pages 29-32; FDA, 2015a)
Wistar Crl:WI (Han) juvenile rats	0, 2,000, 5,000, or 6,000 mg/kg bw/d (99% purity on a dry weight basis; source-Glycom, synthetic 2'-FL), or 6,000 mg/kg bw/d FOS (reference)	90 d (from PND 7) with 4 wk recovery period	NOAEL= 5,000 mg/kg bw/d for male and female rats	
Wistar Crl:WI	0, 3, 6, or 10% in diet (purity, 94%; source -	13 wk	NOAEL= ≥ 7,250 mg/kg bw/d	van Berlo et al., 2018

(Han) rats	Glycosyn and Friesland Campina, via genetically engineered *E. coli* K12)	(PND 25 to 115)	(males); ≥ 7,760 mg/kg bw/d (females) or 10% of the diet	(also described in GRN 735, pages 54-55; FDA 2018a)
Crl:CD® (SD) neonatal rats	0, 1,000, 3,000, or 5,000 mg/kg bw/d 2'-FL/DFL (82.5%/9.7% w/w or ~8:1 ratio; source - Glycom,), or 5,000 mg/kg bw/d FOS (reference)	90 d with 4 wk follow-up (from PND 7)	NOAEL=5,000 mg/kg bw/d	Phipps et al., 2018
Domestic Yorkshire crossbred swine – farm neonatal piglets	Control, 200, 500, or 2,000 mg/L 2'-FL (purity, 97.9%; source – Jennewein, via genetically engineered *E. coli* BL21)	21 d from 2 days after birth	Well tolerated up to 2,000 mg/L/d; no treatment-related effects; equivalent maximum doses= 292 mg/kg bw/d (males), 299 mg/kg bw/d (females)	Hanlon and Thorsrud, 2014

DFL=difucosyllactose; FOS= fructooligosaccharides; NOAEL= no-observed-adverse-effect level; PND=postnatal day.

Studies of 2'-FL by Coulet et al. (2014); this study was also presented in GRN 546 (pages 29 to 32 - FDA, 2015a).

In a 14-day oral tolerability and dose-range finding study, 2'-FL produced by chemical synthesis (source – Glycom A/S' [Glycom's] synthetic 2'-FL; >99% purity on a dry weight basis) was administered by gavage to 7-day-old Wistar IGS:Crl:WI (Han) rats (n = 5/sex/group) at doses of 0, 2,000, 5,000, or 7,500 mg/kg bw/day (GRN 546, FDA, 2015a; Coulet et al., 2014). A reference control group was administered at 7,500 mg fructooligosaccharides (FOS)/kg bw/day during the 14-day study period starting PND 7 to weaning (PND 21). Observations included food intake, general health, clinical signs, mortality, and morbidity. All animals in the 5,000 and 7,500 mg per kg bw per day groups and in the FOS control group had lower body weight gains between days 0 to 3 as compared with the vehicle control group. The authors concluded that the highest suitable dose of 2'-FL for the 90-day study that followed was lower than 7,500 mg/kg bw/day. Based on the results of this study, the highest dose of the subchronic toxicity study that followed was set at 6,000 mg/kg bw/day.

Subsequently, a 90-day subchronic oral toxicity study of 2'-FL with a 4-week recovery period was conducted starting with 7-day-old Wistar [Crl:Wl(Han)] rats (Coulet et al., 2014). 2’-FL was administered via gavage in a juvenile adapted subchronic rat study at dose levels of 0, 2,000, 5,000, or 6,000 mg/kg bw/day. Fructooligosaccharides at a daily dose of 6,000 mg/kg bw was used as a reference high-dose control. No treatment-related adverse effected were noted. The exception was that one male and one female rat in the 6,000 mg/kg bw/day 2'-FL dose group, and two males and one female in the 6,000 mg/kg bw/day FOS dose group died during the treatment period. One female in the 6,000 mg/kg bw/day FOS group died during the recovery period. Oral administration up to 5,000 mg/kg bw/day to rats over 90 days was not associated with any adverse effects based on clinical observations, body weight gain, food consumption, ophthalmoscopy, clinical pathology, organ weights, and histopathology findings, although transient lower body weight gains and colored/liquid feces were observed during the first few days of the administration period. The authors stated that the deaths in the highest dose group could not be definitely determined to be not treatment-related, and they concluded that the NOAEL for 2'-FL was 5,000 mg/kg bw/day in Wistar [Crl:Wl(Han)] rats.

However, the EFSA NDA Panel evaluated the same study and determined a NOAEL of 2,000 mg/kg bw/day based on decreased relative kidney weight in the high-dose female group, two unexplained deaths, and clinical chemistry effects in the high- and mid-dose groups (EFSA, 2015). Glycom defended their NOAEL of 5,000 mg/kg bw/day because the slight reductions in red blood cell counts pointed out by EFSA were not associated with histopathological or gross pathological correlates and, thus, were not considered treatment-related adverse effects. In addition, aspartate aminotransferase (AST) reductions that EFSA was concerned about were comparable between the mid-dose and control values and, thus, were not considered adverse.

A 90-Day Oral Toxicity Study of 2’-FL by van Berlo et al. (2018); the same study was presented in GRN 735, pages 54 to 55 - FDA, 2018a)

In a study by van Berlo et al. (2018), 2′-FL (94% purity; source, Glycosyn and FrieslandCampina; produced through fermentation by genetically modified *E. coli* K12) was administered to Wistar Han IGS rats [Crl:WI(Han)] for 13 weeks from post-natal days 25 to 115. The concentrations of 2’-FL in the diet were 0% (control), 3%, 6%, and 10%. These levels correspond to 2,170, 4,270, and 7,250 mg/kg bw/day for males and 2,450, 5,220, and 7,760 mg/kg bw/day for females, respectively. No treatment-related adverse effects of 2’-FL were observed in general condition, weight gain, neurobehavioral observations, ophthalmoscopy, hematology, clinical chemistry, urinalysis, organ weights, or in macroscopy and microscopy of organs and tissues. The

exception was overall food consumption that was slightly decreased in the high-dose female group (control vs. high-dose group, 14.1 vs. 13.1 g/rat/day). Because the relative difference with controls was small (less than 10%) and no clear corroborative changes were observed in other parameters including growth, this finding was considered to be of little toxicological significance. Thus, the NOAEL was determined to be the highest levels tested: 7,250 mg/kg bw/day for male rats and 7,760 mg/kg bw/day for female rats.

A Piglet Study of 2’-FL by Hanlon and Thorsrud (2014); the same study was summarized in GRN 571 (stamped pages 43 and 44 - FDA, 2015b)

In a piglet study, 2’-FL produced by fermentation using *E. coli* BL21 (94.1% purity; source, Jennewein Biotechnologie) was administered by gavage to neonatal pigs at concentrations of 0, 200, 500, or 2,000 mg/L for 20 days from day 2 of lactation (Hanlon and Thorsrud, 2014). These levels corresponded to dose levels of 29.4, 72.2, or 292 mg/kg bw/day in males and 29.3, 74.3, or 299 mg/kg bw/day in females, respectively. There were no treatment-related effects on growth, clinical observations, body weight, food consumption, hematology, clinical chemistry, coagulation, urinalysis, or any histopathologic changes. Therefore, the authors concluded that dietary exposure to 2′-FL at concentrations up to 2,000 mg/L (up to 292 mg/kg bw/day in males and 299 mg/kg bw/day in females) was well tolerated and supported normal growth patterns in neonatal piglets with no adverse effects (Hanlon and Thorsrud, 2014).

A Study of the Mixture of 2’-FL and DFL by Phipps et al. (2018)

In the subchronic study by Phipps et al. (2018), a mixture of 2′-FL and difucosyllactose (DFL) (82.5%; DFL, 9.7%; manufacturer-Glycom) was administered to Crl:CD®(SD) rats once daily for 90 days starting post-natal day (PND) 7, followed by a 4-week recovery period. Doses tested were 0, 1,000 (low-dose), 3,000 (mid-dose) or 5,000 (high-dose) mg/kg bw/day of a 2’-FL/DFL mixture or 5,000 mg/kg bw/day of fructooligosaccharide (FOS) as a reference. The authors determined the NOAEL as 5,000 mg/kg bw/day based on the absence of treatment-related adverse effects in this 90-day study.

The European Food Safety Authority Dietetic Products, Nutrition and Allergies (EFSA NDA) Panel (EFSA, 2019a) evaluated the same study and did not establish a NOAEL of the test substance (an 8:1 mixture of 2’-FL/DFL). The EFSA NDA Panel noted significant differences in several hematological and clinical chemistry parameters. Examples included increased hematocrit, hemoglobin, and red blood cell count (RBC), as well as decreased mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) for all 2′-FL/DFL male groups, increased basophils and large unstained cells (LUC) for all 2′-FL/DFL male groups, increased basophils, decreased

monocytes, and reduced albumin concentrations for all 2′-FL/DFL female groups. The panel also noted significant differences observed in several hematological and clinical chemistry parameters that were often seen only in the low- and mid-dose groups while differences between the high-dose and control groups were minor or not statistically significant. For example, aspartate transaminase [AST] levels were significantly higher only in low-dose and middle-dose male groups and low-dose female groups while high-dose and control groups had comparable values. Similar examples include, but are not limited to, increased hematocrit, hemoglobin, and red blood cell count (RBC); mean cell volume (MCV) for the low- and mid-dose females; increased MCV for mid-dose males and females; decreased red cell distribution width (RDW) for low- and mid-dose males; decreased mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC), white blood cell count (WBC), lymphocytes, and eosinophils for low- and mid-dose females; decreased red cell distribution width (RDW) for low- and mid-dose males; increased aspartate aminotransferase (AST) for low- and mid-dose male groups and females given a low-dose; and reduced albumin for the mid-dose male group. The EFSA NDA Panel also noted no macroscopic or histopathological findings in the treated groups. The Panel stated that they could not establish a NOAEL of the test substance from this 90-day oral toxicity study because they could not interpret the biological relevance and adversity of the significant differences in the observed hematological and clinical chemistry parameters, which did not show a dose–response relationship.

However, Glycom defended the NOAEL of 5,000 mg/kg bw/day for a mixture of 2′-FL and DFL in its GRAS notification (FDA, 2019c), as all individual values for all of these hematological and clinical chemistry parameters were within respective historical control data ranges. The data indicated that all the hematological and clinical chemistry values were within normal biological variation.

6.C.3.2. Unpublished Corroborative Animal Toxicity Studies

Because the specifications and composition for APTech’s 2′-FL in this GRAS assessment are substantially equivalent to those described in published studies by Coulet et al. (2018) and van Berlo et al. (2018) and APTech’s 2′-FL is structurally identical to that studied in Coulet et al. (2018), it is reasonable to expect that the NOAEL of APTech’s 2’-FL would be at least 5,000 mg/kg bw/day.

Findings from unpublished 90 day oral toxicity studies in rats were consistent with those reported in published studies. As shown in Tables 16-2, various purified 2’-FL preparations showed similar toxicology profiles, regardless of methods of manufacture.

Case and Yoon (2020, unpublished; Appendix J) reported the NOAEL of 7,500 mg/kg bw/day in both male and female rats for APTech’s 2’-FL prepared by fermentation with genetically modified *C. glutamicum* APC199. Jennewein

Biotechnologie (GRN 571 - FDA, 2015b) reported the NOAEL of 7,660 and 8,720 mg/kg bw/d for male and female rats, respectively, for the 2'-FL preparation manufactured via genetically modified *E. coli* BL21. Penard (2015, GRN 650 - FDA, 2016a) reported that the NOAEL for 2'-FL manufactured via genetically modified *E. coli* K12 was 5,000 mg/kg bw/d for both male and female rats. For all purified 2'-FL preparations, the NOAELs were determined to be at least 5,000 mg/kg bw/day in rats, regardless of methods of manufacture. Overall, these corroborative data support the findings from published studies. Thus, the unpublished status of these studies has no impact on the overall conclusion of this GRAS determination if qualified experts do not have access to such data and information.

An Acute Toxicity Study of APTech's 2'-FL

Table 16-1 summarizes the results from an acute oral toxicity study conducted with APTech's 2′-FL (purity, ≥94%) in juvenile (7 day old) male and female Sprague-Dawley rats (Case and Yoon, 2020, unpublished; Appendix J). The test groups consisted of three dose groups at dose levels of 2,500, 5,000, and 7,500 mg 2′-FL/kg bw and a control group (water), with 5 animals of each sex per group. All animals were monitored for clinical signs and body weight changes during the 14-day observation period after dosing. They were euthanized and subjected to gross necropsy at the end of the observation period. One female was found dead at 7,500 mg/kg bw on day 2 after dosing. However, there were no test substance-related clinical signs and body weight changes in the other female pups in the 7,500 mg/kg bw dosing group. It was not considered to be a test substance-related mortality. There were no abnormalities in clinical signs for all groups. During the 14 day observation period, the body weight gain was significantly suppressed in the high dose male group, but not in females (control vs. low- vs. mid- vs. high-dose: males - 53.1 vs. 50.5 vs. 51.5 vs. 45.0 g, $P<0.01$; females - 50.5 vs. 49.0 vs. 48.0 vs. 47.0 g, NS).

At necropsy, there were no test substance-related gross findings in either sex at 2,500, 5,000, and 7,500 mg/kg. It was concluded that the mean lethal dose (LD_{50}) was greater than 7.5 g/kg bw, the highest dose tested. A compound that has a LD_{50} value of over 5 g/kg bw in rats is classified as 'practically nontoxic' (Altug, 2003).

Table 16-2. Summary of Unpublished Corroborative Toxicity Studies

Animal	2'-FL Dose	Duration	Results	Reference
Crl:CD(SD) Rat	0, 2.5, 5, or 7.5 g/kg bw (purity, ≥94%; source - APTech, produced via genetically modified *C. glutamicum* APC199)	Single dose; 14 d observa-tion	Mean lethal dose (LD_{50}) was greater than 7.5 g/kg bw; lower body wt. gain in the high-dose group	Case and Yoon, 2020 (Appendix J)
Crl:CD(SD) rats	0, 2.5, 5, or 7.5 g/kg bw (purity, ≥94 %; source - APTech, via genetically modified *C. glutamicum* APC199)	90 d with 4 wk follow-up (from PND 7)	NOAEL=7,500 mg/kg bw/d for male and female rats, the highest dose tested	Case and Yoon, 2020 (Appendix J)
Female Crl:CD(SD) rats	0 or 10% in diet (purity, 94.1%; source - Jennewein, via genetically modified *E. coli* BL21	7 d (from PND 59)	No treatment-related abnormalities	GRN 571 (unpublished; stamped pages 42-44; Appendix M3, p 473 - FDA, 2015b)
Crl:CD(SD) rats		90 d (from PND 26 [m] or 28 [f])	NOAEL= 7,660 mg/kg bw/d (males); 8,720 mg/kg bw/d (females)	
Wistar Crl:WI (Han) rats	0, 2,000, 4,000, or 5,000 mg 2'-FL/kg bw/d (purity, 97.6%; source - Glycom, via genetically modified *E. coli* K12), or 5,000 mg/kg bw/d FOS (reference)	90 d with 4 wk recovery period (from PND 7)	NOAEL= 5,000 mg/kg bw/d for male and female rats	Penard, 2015 (Unpublished; GRN 650, stamped pages 37-39; FDA, 2016a)

F=females; m=males; NOAEL= no-observed-adverse- effect level; PND=postnatal day.

An Acute Toxicity Study of APTech's 2'-FL, continued

In the 90 day oral toxicity study in rats, a transient body weight decrease was noted in males in the 5,000 and 7,500 mg/kg/day groups on days 11 and 4, respectively. These changes were not considered test substance-related because there was no dose dependency and they were temporary, with little difference compared to the control group. Starting day 15 after dosing, no significant differences in body weights were observed in either sex among the 4 groups. Overall, the evidence does not support that 2'-FL consistently reduced body weight gain. Even if 2'-FL can

consistently reduce body weight gain, it may not be considered an adverse effect. It is due to the fact that non-digestible carbohydrates, in general, have a tendency to prevent weight gain which is not considered an adverse effect (Slavin, 2008).

Subchronic Oral Toxicity Study of APTech's 2'-FL

This study was conducted to assess the potential toxicity and safety of the test substance, 2'-FL (purity, ≥94%), when administered by gavage once daily to juvenile Sprague-Dawley [Crl:CD(SD)] rats of both sexes for 90 days with a 28 day recovery period (Table 16-1; Case and Yoon, 2020– unpublished; Appendix J). On postnatal day (PND) 7, animals (10/sex/group) were assigned to one of the 4 groups (0, 2,500, 5,000, or 7,500 mg /kg bw/day). At post-weaning on PND 21, all pups were separated individually in each cage. An extra 5 animals of each sex were added to the control group and the 7,500 mg/kg bw/day group for the recovery groups to assess the reversibility of toxicity during the 4-week recovery period. Evaluated parameters included clinical signs, detailed examinations, body weight, food consumption, functional observations, ophthalmological examinations, urinalysis, hematological and clinical chemistry examinations, organ weights, gross post-mortem examinations, and histopathological examination.

One male of the 5,000 mg/kg/day group was found dead on day 72. Based on the absence of morphological changes, no apparent effects at necropsy or histopathological lesions, it was considered to be a sudden death that often occurs in Sprague-Dawley rats. One female of the 7,500 mg/kg/day group was found dead on day 26. Serous fluid-filled thoracic cavity (clear with red color) and pulmonary congestion/edema were noted in the dead female. The GLP laboratory that conducted the study reported that these findings might be due to a technical gavage error.

During the dosing period, there were statistically significant decreases in body weight in males in the 5,000 mg/kg bw/day group on Day 11 (47.5±2.8 vs. 44.7±2.6 g, $P<0.05$) and the 7,500 mg/kg/day group on Day 4 (27.6±1.7 vs. 26.0±0.8 g, $P<0.05$). These changes were not considered test substance-related because there was no dose dependency and they were temporary, with little difference compared to the control group. Starting day 15 after dosing, no significant differences in body weights were noted in either sex among the 4 groups (control vs. low- vs. mid- vs. high dose: males at day 90, 602.2 vs. 625.1 vs. 581.5 vs. 647.2 g, NS; females at day 90, 337.7 vs. 323.4 vs. 325.9 vs. 321.5 g, NS). During the recovery period, there was no test substance-related effect in both sexes in the 7,500 mg/kg/day group.

Overall, no test substance-related abnormalities were noted in clinical signs, body weights, food consumption, functional observations, ophthalmological

examination, urinalysis, hematology, clinical chemistry, organ weights, and gross postmortem examinations in males and females in the 2,500, 5,000, and 7,500 mg/kg bw/day groups. No test substance-related effects were noted in the histopathological examination of males and females in the 7,500 mg/kg bw/day group. Thus, the NOAEL of APTech’s 2’-FL was determined to be 7,500 mg/kg bw/day in both male and female rats after repeated oral administration for 90 days under the conditions of this study. Details are presented in Appendix J.

A 90-Day Oral Toxicity Study Presented in GRN 571 (pages 42 to 43 - FDA, 2015b)

GRN 571 describes a 90-day study (unpublished) in which 4-week-old CD® rats [Crl:CD(SD), n=10/sex/group] were fed a standard rat diet *ad libitum* (control) or the standard rat diet that was supplemented with 0 or 10% of 2'-FL prepared via fermentation using genetically modified *E. coli* BL21 (source - Jennewein; 94.1% purity; specification >90%) (GRN 571; FDA, 2015b). An additional 3 animals per sex in the control group and 9 animals per sex in the treatment group were used exclusively for blood sampling. No treatment-related abnormalities were observed in feed intake, clinical signs, body weight, organ weights, behavior, appearance, hematology, clinical biochemistry, urinalysis, ophthalmological examination, or histopathological examinations. The study authors concluded that the NOAEL of 2'-FL was determined to be at least 7,660 and 8,720 mg/kg bw/day in male and female rats, respectively.

A 90-Day Oral Toxicity Study Presented in GRN 650 (stamped pages 37 to 39; summary on page 68 - FDA, 2016a)

Penard (2015; unpublished) conducted a 90-day oral toxicity study with an additional 28 day recovery period in Wistar [Crl:WI(Han)] rats on 2'-FL (source- Glycom; 97.6% purity, produced through fermentation using genetically modified *E. coli* K12; FDA, 2016a). In the main study, seven-day old neonatal Wistar rats were administered 2,000, 4,000, or 5,000 mg/kg bw of Glycom's 2'-FL or 5,000 mg/kg bw/day of FOS (reference group) for 90 days. Animals in the recovery group (5 rats per sex) were also administered control, 2'-FL, or FOS for 90 days after which they remained untreated for 28 days. One dam was then housed with a reconstituted litter of 5 pups per sex, fed a standard diet, and the pups were treated with the same dose of 2'-FL starting postnatal day (PND) 7 to weaning on PND 21. On PND 21, pups were weaned and placed in plastic cages according to sex and dose group. A total of 5 pups of the same sex and dose group were housed per cage. No deaths were associated with the test item. Liquid feces were noted for most rats that were treated with FOS and for animals in the mid- and high-dose 2'-FL groups. No treatment-related abnormalities were observed in food intakes, body weights, organ weights, clinical chemistry, urinalysis, and macroscopic or histological observations. The authors determined a NOAEL of 5,000 mg/kg bw/day for 2’-FL.

Conclusion of Animal Toxicity Studies

From the two published studies of purified 2'-FL (synthetic 2'-FL and a preparation manufactured via genetically modified *E. coli* K12), the NOAEL was determined to be 5,000 mg/kg bw/day in rats. Results from unpublished studies confirmed the findings from published studies. It appears that various purified 2'-FL ingredients showed similar toxicology profiles regardless of methods of manufacture. Additionally, the addition of 2'-FL concentrations of up to 2,000 mg/L (corresponding to up to 292 mg/kg bw/day in males and 299 mg/kg bw/day in females, the highest dose tested) was well tolerated and supported normal growth patterns in neonatal piglets with no adverse effects.

The corroborative unpublished data from toxicology studies of APTech's 2'-FL support the findings from published studies. Thus, the unpublished status of the APTech's toxicity studies has no impact on the overall conclusion of this GRAS determination if qualified experts do not have access to such data and information.

6.C.4. Animal Efficacy Studies

Since the FDA's last review of 2'-FL (GRN 735, pages 59 to 60 - FDA 2018a; GRN 749, pages A110 to A112 - FDA, 2018b; GRN 852, pages 45 to 46 - FDA, 2019c), two animal efficacy studies were published. These efficacy studies showed that 2'-FL did not result in any adverse effects on immune responses (van den Elsen et al., 2019; Xiao et al., 2018). The results are summarized in Table 17. Neither of these studies reported adverse effects of 2'-FL on measured outcomes.

Recent Animal Efficacy Studies

Since the FDA's last review of 2'-FL, two animal study reported the repeat dose administration of 2'-FL at high dietary concentrations (Table 18). Any studies using modified genes or chemically- or biologically-induced disease models were not included in this review because the data from these induced disease conditions may not be relevant when evaluating the safety of 2'-FL.

Xiao et al. (2018) determined the effect of 2'-FL on vaccination responsiveness (both innate and adaptive) in a murine influenza vaccination model in 6-week-old female C57BL/6JOlaHsd mice. Dietary 2'-FL (0.25 - 5.0%, w/w) was administered to mice for 31 days starting from two weeks prior to primary and booster vaccination (intradermal challenge). Measurements included vaccine-specific cellular and humoral response (serum vaccine-specific IgG1 and IgG2a), B-cell activation, activated splenic dendritic cells and mesenteric lymph nodes, splenic Th1 and Tregs frequency, and vaccine-specific CD4+ and CD8+ T-cell proliferation. No adverse effects were reported on measured outcomes.

In a study by van den Elsen et al. (2019), mice were supplemented with a mixture (2% in diet) of 2'-FL (>90% purity), short-chain galactooligosaccharides (scGOS), and long-chain fructooligosaccharides (lcFOS) from different stages in early life. BALB/c breeding pairs were fed either a control diet (AIN93G) from the day of timed mating or an oligosaccharide diet (2% of the diet; AIN93G containing the mixture of 2’-FL/scGOS/lcFOS, the ratio of each oligosaccharide was not specified). A third of the breeding pairs that received the control diet from the day of mating were switched to the diet with the mixture of oligosaccharides within 24 h after birth and, after weaning, their litters were maintained on the oligosaccharides diet throughout the course of the experiment. The litters from another third of the control breeding pairs were provided the oligosaccharide diet at weaning and maintained on this diet throughout the experiment. At 6 weeks of age, male and female offspring of all 4 dietary groups were immunized subcutaneously with trivalent influenza vaccine. Measurements included development of the gut microbiota and antibody-mediated vaccine responses. No adverse effects of the oligosaccharide mixture were observed on measured outcomes.

Conclusions from Animal Efficacy Studies

Doses up to 5% 2’-FL in diet were well tolerated in mice with no adverse effects.

Table 17. Summary of Recent Animal Efficacy Studies of 2'-FL

Objective	Animal	Dose	Duration	Measurements	Reference
The Studies Reviewed in this GRAS Notice					
To determine the effect of 2'-FL on vaccination responsiveness (both innate and adaptive) in a murine influenza vaccination model	Female C57BL/6JOlaHsd mice, vaccinated with inactivated influenza virus vaccine	Control, 0.25, 0.5, 1, 2.5, or 5% 2'-FL in diet; 2'-FL source-SSNIFF Spezialdiäten; purity >90%	31 d	Vaccine-specific cellular and humoral response (serum vaccine-specific IgG1 and IgG2a); B-cell activation and frequency; activated splenic dendritic cells and mesenteric lymph nodes; splenic Th1 and Tregs frequency; vaccine-specific CD4+ and CD8+ T-cell proliferation	Xiao et al., 2018
To determine the effect of the oligosaccharide mixture including 2'-FL on the gut microbiota and antibody-mediated vaccine responses	Male and female BALB/c mice, vaccinated with trivalent influenza vaccine	AIN 93G control diet or a mixture of 2'-FL/short-chain GOS/ and long-chain FOS, 2% (w/w) of the diet (the ratio of each oligosaccharide, not specified)	Different stages in early life	Development of the gut microbiota and antibody-mediated vaccine responses	van den Elsen et al., 2019

N= number of animals per group; FOS= fructooligosaccharide; GOS= galactooligosaccharide.

6.C.5. Human Clinical Studies

Since the FDA’s last review of 2’-FL (GRN 735, pages 60 to 63 - FDA 2018a; GRN 749, pages 37 to 39 - FDA, 2018b; GRN 852, pages 48 to 49 - FDA, 2019c), two new human studies were published (Table 18; Nowak-Wegrzyn et al., 2019; Storm et al., 2019). Because APTech’s 2’-FL in this notice is substantially equivalent to those described in previous GRAS notices, the safety data and discussion presented in previous GRAS notices are also applicable when evaluating the safety of APTech’s 2’-FL. This information is hereby incorporated, by reference, in this document and will not be discussed in detail. For these ‘pivotal’ studies, the levels of consumption represent the maximum dose consumed rather than absolute safety endpoints.

6.C.5.1. Recent Human Clinical Studies

Nowak-Wegrzyn et al. (2019) evaluated clinical hypoallergenicity and safety of an extensively hydrolyzed whey-based infant formula containing 2’-FL (1.0 g/L) and lacto-*N*-neotetraose (LNnT; 0.5 g/L), in infants and children with cow’s milk protein allergy. The control formula did not contain 2’-FL and LNnT. Infants and children with cow’s milk protein allergy (aged 2 months to 4 years) were subjected to food challenges to both formulas in a blinded, cross-over design. Any allergic signs or symptoms (cutaneous, gastrointestinal, respiratory, or cardiovascular) attributable to the challenge formula were assessed. Subjects who successfully passed both sessions of the food challenge participated in a one-week, open food challenge with the test formula. Symptoms and adverse events were recorded. No adverse effects of the test formula were reported in infants and young children with cow’s milk protein allergy.

Storm et al. (2019) evaluated the feeding tolerance of 2'-FL (0.25 g/L) in a 100% whey, partially hydrolyzed infant formula with the *Bifidobacterium animalis* ssp. *lactis* strain Bb12 ($1x10^6$ CFU/g powder) as compared with the control formula without 2'-FL (control) in healthy, full-term infants. Infants, who were enrolled at 2 weeks of age, were fed one of the two formulas for 6 weeks to evaluate various safety parameters. Primary endpoint values, tolerance as assessed by Gastrointestinal Symptom Questionnaire scores, were comparable between groups (test vs. control: 20.9 vs. 20.7, NS). The secondary endpoints included stooling, vomiting, spit-up, crying, and fussing. The incidences of other adverse effects/events were comparable between the groups. The authors concluded that partially hydrolyzed, whey-based infant formula containing 2'-FL and *B. lactis* was tolerated well.

6.C.5.2. Human Clinical Studies Included in Previous GRAS Notices

As shown in Table 18, studies evaluated the effects of 2’-FL on various measurement outcomes in infants including growth and tolerance (Marriage et al., 2015; Puccio et al., 2017), 2’-FL absorption and excretion (Marriage et al., 2015), formula

intake, behavioral patterns, and morbidity including parents-reported adverse events and concomitant medications (Puccio et al., 2017), global average microbial composition profile (Steenhout et al., 2016), and markers of immune functions (Goehring et al., 2016). Healthy infants received daily doses of up to 1.0 g of 2’-FL/L for up to 6 months (Marriage et al., 2015; Goehring et al., 2016; Puccio et al., 2017; Steenhout et al., 2016). No adverse effects of 2’-FL were reported on the measured outcomes listed above.

A human study in adults evaluated the effect of 2’-FL on safety including gastrointestinal symptoms, clinical chemistry, hematology, and gut microbiota (Table 20; Elison et al., 2016). Healthy adults received 2’-FL or lacto-*N*-neotetraose (LNnT) doses up to 20 g/day, either alone or in combination for up to 2 weeks. The subjects were randomized to one of 10 groups: 2′FL, LNnT or the mixture of 2′FL and LNnT (2:1 mass ratio) at 5, 10 or 20 g per day or 2 g of glucose as placebo. Hematological and blood biochemistry analyses, fecal microflora and biomarkers (calprotectin, secretory IgA, and short chain fatty acids levels), and adverse events were compared to the baseline values. Adverse events were related mainly to gastrointestinal symptoms, particularly gas/flatulence, and were generally not significant in the low- and mid-dose groups. However, volunteers taking the high 20 g dose of 2′-FL reported increased bloating, flatulence, rumbling, nausea, and diarrhea as well as loose stools and urgency to pass stools. Compared to the control group, the high dose 2’-FL or LNnT groups (20 g/day) had significantly higher Bristol Stool Form Scale (BSFS) scores, indicating softer stools. However, the differences were small (<0.5 point increase). The authors stated that the clinical chemistry and hematology parameters remained within the normal ranges throughout the intervention (data were not presented in this study). Compared to the glucose control, the mixture of 2’-FL and LNnT had no significant effects on stool frequency and consistency.

Consumption of a large amount of non-digestible carbohydrates is often associated with gastrointestinal distress (Institute of Medicine [IOM], 2002). The IOM states that “Although occasional adverse gastrointestinal symptoms were observed with the consumption of dietary and functional fibers, serious chronic adverse effects have not been observed.”

Summary of Human Clinical Studies

Various 2’-FL preparations in relatively high purity were proven safe in infants: formulas supplemented with 1.0 g/L 2’-FL were well tolerated in infants. In adults, daily doses of up to 10 g/day were considered to be safe.

Table 18. Summary of Infant Studies of 2′-FL

Subject	Dose	Duration	Measurements	Reference
Recent Studies with Infants				
67 infants and children with cow's milk protein allergy (N=31-36)	2 groups: control or test formula containing 1.0 g 2'-FL/L and 0.5 g LNnT/L (2'-FL source-NA, purity-NA)	1 wk	Any allergic signs or symptoms (cutaneous, gastrointestinal, respiratory, or cardiovascular) and adverse events	Nowak-Wegrzyn et al. (2019)
79 healthy, full-term infants (N=39-40)	2 groups: 0 or 0.25 g 2'-FL/L (2'-FL source-NA, purity-NA) with *B. lactis* Bb12	6 wk from 2 wk of age	Adverse events; safety parameters including gastrointestinal tolerance, stooling, vomiting, spit-up, crying, and fussing	Storm et al., 2019
Studies with Infants Reviewed in Previous GRNs*				
175 healthy infants (N= 87-88)	2 groups: Control formula; or formula supplemented with 1.0 g/L 2'-FL + 0.5 g/L LNnT (2'-FL source, Glycom, purity-NA)	From ≤14 d to 6 mo of life; Follow-up formula with no HMOs from 6 to 12 mo of life	Weight gain through 4 mo of age; anthropometric measures, gastrointestinal tolerance, stool characteristics, formula intake, behavioral patterns, and morbidity through 12 mo of age	Puccio et al., 2017
161 healthy term infants (N=38-65)	3 groups: Control formula; test (formula + 1.0 g/L 2'-FL + 0.5 g/L LNnT); or breast-fed (2'-FL source-NA)	From ≤14 d to 3 mo of age	Global average microbial composition profile	Steenhout et al., 2016 (abstract)
420 healthy, full-term infants (N= 101-109)	4 groups: 2.4 g/L GOS control (CF); 2.2 g/L GOS + 0.2 g/L 2'-FL; 1.4 g/L GOS + 1.0 g/L 2'-FL; or breast-fed reference group	From ≤5 to 119 d of life	Growth (weight, length, head circumference); safety (parents reported adverse events); gastrointestinal tolerance; relative absorption and excretion of 2'-FL including 2'-FL concentrations in blood and urine	Marriage et al., 2015

201 healthy term infants (N=48-51; N for blood analysis = 37-42)	(2’-FL source-NA, probably manufactured by fermentation), purity-NA)	Enrolled by day 5 of life; blood drawn at 6 wk of age (Cohort of Marriage et al., 2015)	Markers of immune function: inflammatory cytokine profiles in plasma and peripheral blood mononuclear cells; immune cell proliferation; circulating lymphocyte populations	Goehring et al., 2016

*Infant studies employing 2’-FL concentrations of 1.0 g/L or higher are included in this review. LNnT= lacto-N-neotetraose; N= the number of subjects in each group; NA= not available; d=day; mo=months; wk=weeks.

Table 19. Summary of Human Adult Clinical Studies of 2′-FL Reviewed in Previous GRNs

Subject	Dose	Duration	Measurements	Reference
Studies with Adults				
Healthy adults (mean age 29.3-39.9 y; N=10)	10 groups: Placebo (2 g/d glucose); 2’-FL (source-Glycom chemically produced; 99.9% purity, dw basis), LNnT, or 2’-FL + LNnT (2:1 mass ratio) at 5, 10, or 20 g/d	2 wk	Safety and tolerance (Gastrointestinal Symptom Rating Scale; clinical biochemistry and hematology); fecal microbiota and bacterial metabolites	Elison et al., 2016

dw= dry weight; LNnT=lacto-N-neotetraose; N= the number of subjects in each group.

6.D. Potential Allergenicity

It is not likely that 2'-FL would cause adverse allergenic reactions for the following reasons: 1) The protein content in the APTech's 2'-FL is ≤ 100 μg/g or 0.01% (w/w) as indicated in the specifications; 2) none of introduced gene products [GDP-L-fucose synthase (WcaG), GDP-D-mannose 4,6-dehydratase (Gmd), lactose permease (LacY), and α-1,2-fucosyltransferase (α-1,2-ft)] were found to have homology in amino acid sequences with those of allergenic proteins when the allergenic potential was screened using the database, http://allergenonline.org/databasefasta.shtml (March 23, 2018 version); and 3) no production microorganisms and residual DNA were present in APTech's 2'-FL.

6.E. Safety of Production Microorganism and Introduced Gene Products

The *Corynebacterium glutamicum* species is regarded as qualified presumption of safety (QPS) when used as a production organism for amino acids and other food or feed ingredients (EFSA, 2019b).

6.E.1. *In vitro* Safety Tests for *Corynebacterium glutamicum* APC199 Host Strain

The analysis of whole genomic sequencing of APTech's *Corynebacterium glutamicum* APC199 revealed that the strain is absent of virulence genes (Appendix K). In addition, *Corynebacterium glutamicum* APC199 was shown to have the following characteristics:

(1) has no hemolytic activities,
(2) has no gelatinase activities, and
(3) does not produce biogenic amines.

Details are presented in Appendix K.

6.E.2. Safety of Introduced Gene Products

As recommended by FAO/WHO (2001), the allergenic potential for introduced gene products (proteins) was screened using the database, http://allergenonline.org/databasefasta.shtml (March 23, 2018 version).

None of introduced gene products [GDP-L-fucose synthase (WcaG), GDP-D-mannose 4,6-dehydratase (Gmd), lactose permease (LacY), and α-1,2-fucosyltransferase (α-1,2-ft)] codes for amino acid sequences homologous with allergenic sequences. Details are presented in Appendix L.

6.F. Safety Determination

The following safety evaluation fully considers the composition, intake, and microbiological and toxicological properties of 2’-FL as well as appropriate corroborative data.

1. Analytical data from multiple lots indicate that APTech’s 2′-FL ingredient reliably comply with the established specifications and meet all applicable purity standards.

2. The intended uses and use levels of 2’-FL are the same as those described in GRN 735. APTech intends to use 2’-FL as an ingredient in the following food categories:
   1) whey-, milk-, and/or soy-based, non-exempt infant formulas for term infants at a maximum level of 2.4 g/L of formula as consumed (ready-to-drink or reconstituted formula prepared from powder),
   2) formulas for toddlers and children aged 12 to 36 months at a maximum level of 2.4 g/L of formula as consumed (ready-to-drink or reconstituted formula prepared from powder),
   3) foods for infants and toddlers at levels of 0.24 - 1.2 g/serving, and
   4) the following food categories at levels of 0.28 - 1.2 g/serving: 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 and milk products, processed fruits and fruit juices, and sweet sauces, toppings, and syrups.

3. From the use of 2’-FL in only infant formulas (2.4 g/L of ready-to-drink or reconstituted formula prepared from powder), in all-user infants aged 0 to 11.9 months old, the estimated mean and 90th percentile intakes of 2’-FL were determined to be at 1.87 and 2.78 g/person/day, respectively. On a body weight basis, these intakes were determined to be 258.7 and 431.3 mg/kg bw/day, respectively. The all-user estimated mean and 90th percentile intakes of 2’-FL were greatest in infants aged 3 to 5.9 months old at 2.04 and 2.93 g/person/day, respectively. On a body weight basis, the greatest intake was observed to occur in infants aged 0 - 2.9 months at 347.8 and 541.9 mg/kg bw/day, respectively.

   Under the intended use of 2’-FL in infant formula and other foods, the mean and 90th percentile EDIs of 2'-FL in all-users of all ages were determined to be 1.70 and 3.54 g/person/day, respectively. On a body weight basis, the mean and 90th percentile EDIs were determined to be 36 and 80 mg/kg bw/day, respectively, in all-users of all ages. The highest intake was observed to occur in male teenagers with the highest 90th percentile intake at 4.29 g/person/day. Of all-users, infants

aged 0 to 5.9 months were estimated to have the highest mean and 90th percentile EDIs of 315 and 532 mg/kg bw/day, respectively. These EDIs are within safe intake levels.

4. Human infants have been exposed to 2’-FL via human breast milk. The EDIs of the 2’-FL for the proposed uses at their respective maximum use levels are unlikely to exceed the high intake level of 2’-FL in breastfed infants per kg bw because the maximum use level of 2’-FL in infant formula is similar to the mean concentration of 2’-FL in human milk (approximately 2.4 g/L). Thus, the intake of 2’-FL for the proposed uses at their respective maximum use levels can be considered safe.

5. Because APTech’s 2’-FL will replace other 2’-FL ingredients in the marketplace, an increase in cumulative intake is not expected.

6. Because the specifications and composition for APTech’s 2′-FL in this notice are substantially equivalent to those described in previous GRAS notices, the safety data and discussion in these GRAS notices are also applicable to the evaluation of the safety of APTech’s 2′-FL. Various purified 2’-FL preparations showed similar toxicology profiles regardless of manufacturing methods. For all 2’-FL preparations, the NOAELs were determined to be over 5,000 mg/kg bw/day in subchronic toxicity studies in rats. The addition of 2’-FL at doses up to 2,000 mg/L was well tolerated and supported normal growth patterns in neonatal piglets.

7. APTech’s 2’-FL is chemically and structurally identical to the 2’-FL found in human milk and, therefore, the safety of APTech’s 2’-FL is supported by the known consumption of 2’-FL from human breast milk in infants. In addition, APTech’s 2’-FL is chemically and structurally identical to synthetic 2’-FL preparation, for which FDA issued a ‘no question’ letter.

8. Human clinical studies showed that 2’-FL was safe in both infants and adults: formula supplemented with 1.0 g/L 2’-FL was well tolerated in infants and daily doses of up to 10 g 2’-FL were shown to be safe in adults.

6.G. Conclusions and General Recognition of the Safety of 2’-FL

6.G.1. Common Knowledge Element of the GRAS Determination

2′-FL is a naturally occurring trisaccharide found in human milk and is, therefore, typically referred to as a human milk oligosaccharide (HMO). The addition of 2’-FL to term infant formulas is consistent with efforts to produce infant formula that closely matches the nutrient composition of human milk. The FDA has issued ‘no question’ letters in response to several GRAS notifications related to the use of 2’-FL in infant formulas and selected conventional foods. Additionally, in all the studies summarized in previous GRAS determinations, there were no significant adverse effects/events or tolerance issues attributable to 2’-FL in both adults and infants. Because this safety evaluation was based on generally available and widely accepted data and information, it satisfies the so-called “common knowledge” element of a GRAS determination.

6.G.2. Technical Element of the GRAS Determination (Safety Determination)

In addition, the intended uses of 2’-FL have been determined to be safe through scientific procedures as set forth in 21 CFR 170.3(b), thus satisfying the so-called “technical” element of the GRAS determination. The 2’-FL that is the subject of this GRAS determination is produced by genetically engineered, non-pathogenic and non-toxigenic *Corynebacterium glutamicum* APC 199 strain, and its purity is over 94%. The raw materials and processing aids used in the manufacturing process are food grade and/or commonly used in food manufacturing processes. APTech’s 2’-FL is chemically and structurally identical to the 2’-FL found in human milk and to the 2’-FL’s that were concluded to be GRAS in previous GRAS notifications. The literature search did not identify safety or toxicity concerns related to 2’-FL.

It is concluded that APTech’s 2’-FL is GRAS under the intended conditions of use on the basis of scientific procedures, and other experts qualified to assess the safety of food ingredients would concur with these conclusions. Therefore, it is excluded from the definition of a food additive and may be marketed and sold for its intended purpose in the US without the promulgation of a food additive regulation under Title 21 of the CFR.

6.H. Discussion of Information Inconsistent with GRAS Determination

The available data and information appear to be consistent with the conclusion of this GRAS determination.

PART 7. REFERENCES

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Appendices

Appendix A. Particle Size Analysis of 2’-FL

1. Objective

To analyze the particle size of the 2’-FL powder.

2. Methods

The measurement was performed with Horiba Partica LA-950V2 (Horiba Instruments Incorporated, Kyoto, Japan), which allowed for the detection of particles in the size range of 10 nm to 3 mm. Samples were dispersed in ethanol solvent for 1 minute in the instrument and then analyzed. Software version [wet] 5.20 was used for analysis and calculation of a cumulative 50% point of diameter (D50).

3. Results

As a result of analyzing the particle size of 2’-FL powder for 3 batches, the average D50 was 38.8 μm.

Table A.1. Summary of particle size distribution analysis results

No.	Batch No.	D50 (μm)
1	2’-FL-CG-011	38.5770
2	2’-FL-CG-012	38.4959
3	2’-FL-CG-013	39.4727
Mean		38.8485
Standard deviation		0.5421

*Signature*_ 

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March 5, 2019

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Appendix B. Production Strain Construction

1. Host strain

Corynebacterium glutamicum is a non-pathogenic bacterium widely used in industrial amino acid production and has been studied for more than 60 years since its discovery as a glutamate-secreting bacterium (Vertes, 2012). *Corynebacterium glutamicum*, which is a gram-positive and non-spore forming bacterium regarded as a GRAS (generally recognized as safe) strain, has been extensively used in the fermentation industries for the production of amino acids and nucleic acid (Date, 2006, Nakayama, 1978).

C. glutamicum ATCC13032 (NCIMB 10025) was isolated from sewage as a producer of glutamate (Kinoshita et al., 1958). While the strain had been taxonomically named "*Micrococcus glutamicus*" and deposited as ATCC 13032 and NCIMB 10025, it was designated as *C. glutamicum* type strain.

C. glutamicum ATCC 13032 is a strain of the *C. glutamicum* species (other names: DSM 20300, IMET 10482, NCIMB 10025, and KCTC 1445). The genome of *C. glutamicum* ATCC 13032 consists of a single circular chromosome 3.3 megabase pairs (Mbp) in size (Meyer, 2003). The guanine plus cytosine (G+C) content of the genome is 53.8%, which is close to that of *E. coli* and at the lower boundary for the taxonomic class of the *Actinobacteria* which is referred to as high G+C Gram-positive bacteria.

2. Transformed plasmid

The pCN01 plasmid (Figure B.1) was constructed by APTech. The replication origin of *Escherichia coli* (*E. coli*) from pUC18 (GenBank L08752.1, 2,686 bp), *nptII* (a kanamycin resistance gene) from pUC19 (GenBank M77789.2, 2,686 bp), and *C. glutamicum* replication of origin from the *Corynebacterium* plasmid pBL1 (GenBank AF092037.1, 4,447 bp) were used for the construction of pCN01 (Figure B.1). The introduced genetic elements of pCN01 and their position the in plasmid are described in Table B.1.

(pCN01, pUC, and pBL1 are plasmid names)

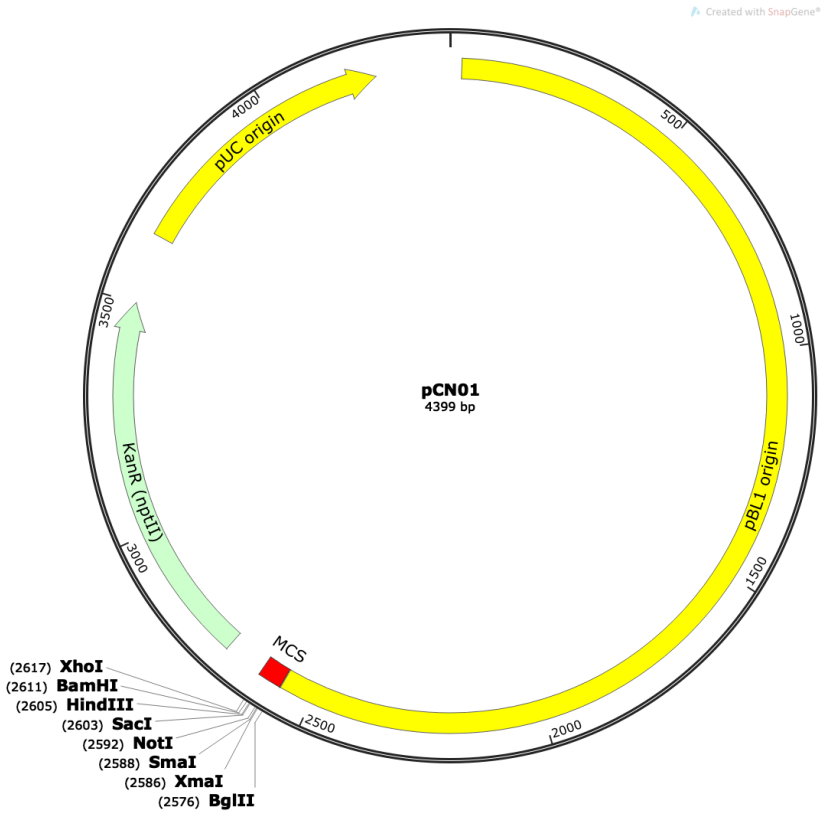

Figure B.1. Plasmid map of pCN01

Table B.1. Descriptions and positions of the introduced genetic elements in the pCN01 plasmid

Component	Function	Position in the plasmid
pBL1 origin	Origin of replication for maintenance in *Corynebacterium glutamicum*	26-2569, 2544 bp
MCS	Multiple cloning site	2571-2621, 51 bp
KanR (*nptII*)	Kanamycin resistance gene	2707-3501, 795 bp
pUC origin	Origin of replication for maintenance in *Escherichia coli*	3651-4239, 589 bp

3. Introduced genes for 2’-fucosyllactose (2’-FL) biosynthesis

Heterologous genes necessary to biosynthesize 2’-FL in *Corynebacterium glutamicum* were inserted into the pCN01 plasmid, resulting in the construction of the pFP110 plasmid (Figure B. 2). The pFP110 plasmid contains four heterologous genes

to enable 2'-FL biosynthesis in *C. glutamicum*. The heterologous genes are α-1,2-fucosyl-transferase (*α-1,2-ft*) [GenBank (Protein): ADY53338.1], GDP-D-mannose-4,6-dehydratase (*gmd*) [GenBank (Protein): AAC75114.1], GDP-L-fucose synthase (*wcaG*) [GenBank (Protein): AAC75113.1], and lactose permease (*lacY*) [GenBank (Protein): AAC73446.1]. APTech's 2'-FL producing strain, designated as *C. glutamicum* APC199, was obtained after transforming the pFP110 into *C. glutamicum* ATCC13032. Overexpression of the above-mentioned genes were achieved by using a strong and constitutive promoter of *C. glutamicum* tuf gene coding for the translational elongation factor EF-Tu. The bacteriophage T7 terminator was used to terminate the transcription. Detailed functions of the introduced genes and their locations in the plasmid pFP110 are summarized in Table B.2. Because the backbone plasmid (pCN01) and the four inserted genes (*α-1,2-ft*, *gmd*, *wcaG*, and *lacY*) have been well-characterized, no unspecified DNA is included in the pCN01 plasmid. The sources of the four heterologous genes are all biosafety level I microorganisms (*E. coli K-12* and *Pseudopedobacter saltans*). A linear map and restriction enzyme sites of pFP110 plasmid are shown in Figure B.3.

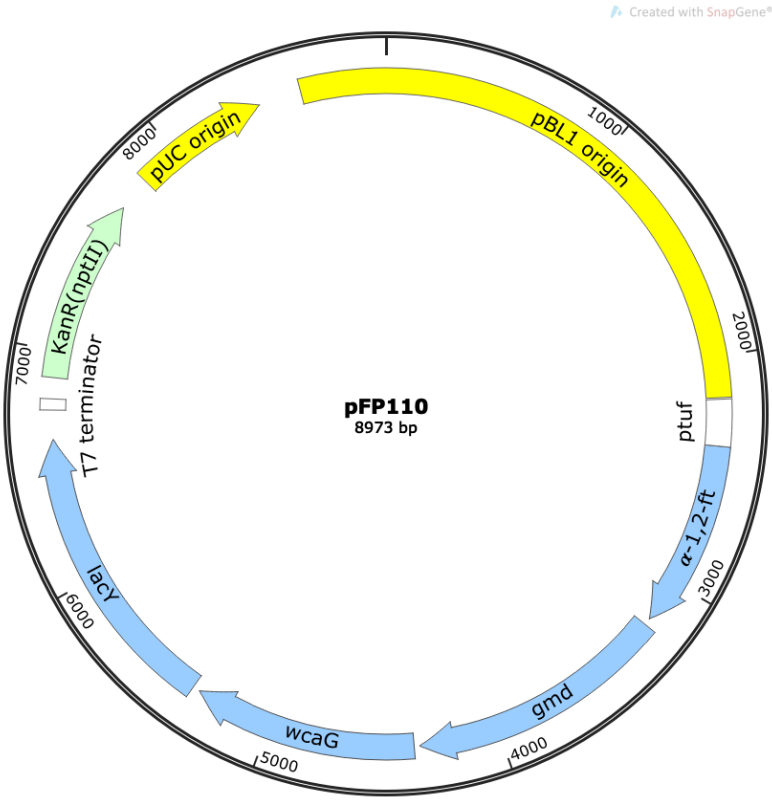

Figure B.2. Plasmid map of the pFP110 containing *α-1,2-ft*, *gmd*, *wcaG*, and *lacY* in a backbone of pCN01

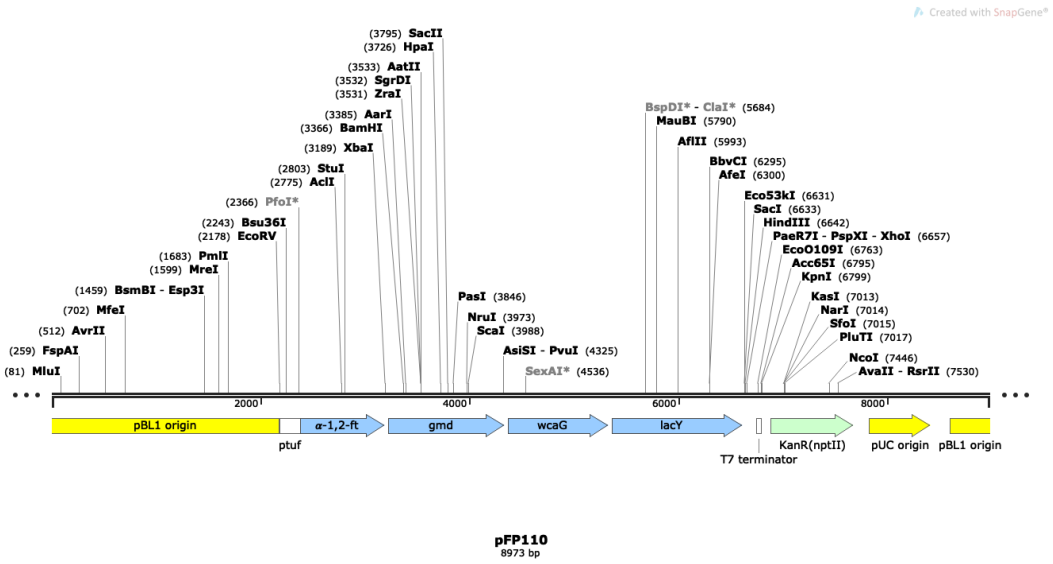

Figure B.3. A linear map and restriction enzyme sites of the pFP110 plasmid

Table B.2. Descriptions and positions of the introduced genetic elements in the pFP110 plasmid

Component	Origin	Function	Position in the plasmid
pBL1 origin	*Corynebacterium* pBL1 plasmid	Plasmid origin of replication in *Corynebacterium glutamicum*	8604-2174, 2544 bp
tuf promoter	*Corynebacterium glutamicum* ATCC 13032	Promoter (transcription start site)	2182-2381, 200 bp
α-1,2-ft	*Pseudopedobacter saltans* ATCC 51119	α -1,2-fucosyl-transferase	2382-3188, 807 bp
gmd	*Escherichia coli* ATCC 700926	GDP-D-mannose-4,6-dehydratase	3220-4341, 1122 bp
wcaG	*Escherichia coli* ATCC 700926	GDP-L-fucose synthase	4367-5332, 966 bp
lacY	*Escherichia coli* ATCC 700926	Lactose permease	5366-6619, 1254 bp
T7 terminator	pET21a plasmid	Transcription termination	6747-6794, 48 bp

KanR (*nptII*)	*Escherichia coli* K-12	Kanamycin resistance gene for antibiotics selection	6886-7680, 795 bp
pUC origin	pUC18 plasmid	Plasmid origin of replication in *Escherichia coli*	7830-8418, 589 bp

References

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Certificate of *Corynebacterium glutamicum* APC199 deposition as KCTC 13735BP

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: **SONG, YoungHa**

Advanced Protein Technologies corp.
5, Mosan-gil, Jeongnam-myeon, Hwaseong-si, Gyeonggi-do
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: *Corynebacterium glutamicum* **APC199**	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: **KCTC 13735BP**
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: [ ] a scientific description [ ] a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **November 23, 2018.**	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **Korean Collection for Type Cultures** Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) 181, Ipsin-gil, Jeongeup-si, Jeolllabuk-do 56212 Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): KIM, Song-Gun, Director Date: **November 23, 2018**

Form BP/4 (KCTC Form 17) sole page

Appendix C. Genetic Stability of pFP110 Plasmid in *Corynebacterium glutamicum* APC199

1. Objective

Recombinant pFP110 plasmid was transformed into *Corynebacterium glutamicum* ATCC13032 and designated as *Corynebacterium glutamicum* APC199. The genes in pFP110 were designed not to be integrated in the host genome but to be inherited in plasmid. In order to verify the absence of non-intentional variations such as addition, insertion, or deletion of the plasmid DNA sequences during multiple generations in an antibiotic-free medium, the entire nucleotide sequences of the plasmid were analyzed by sequencing plasmid DNA extracted from the culture of multiple generations.

2. Materials and methods

1) Sequencing primers

Recombinant pFP110 plasmid were amplified with PCR primers list in Table C.1.

Table C.1. Primers used in this study

Primer name	Primer start position	Sequence (5' -> 3')
FP110seq1-f	213	GTTGCCATGCTTTAAGACTC
FP110seq1-r	1548	CCTTAGACGCGGTTTTAGTA
FP110seq2-f	1356	TTTGAGGATTCCATGTTTTC
FP110seq2-r	2842	TGGTGCCAGGTCTTATAATC
FP110seq3-f	2662	CCAAAGGCTGGTATTTTAGA
FP110seq3-r	4142	TCAACAGCGATAATCACATC
FP110seq4-f	3920	TAAAAATGCAGTGGATGATG
FP110seq4-r	5410	GAATAAACCGAACATCCAAA
FP110seq5-f	5289	CTTACCAGTGGTTCCTTGAG
FP110seq5-r	6619	TTAAGCGACTTCATTCACCT
FP110seq6-f	6415	TCTGGTCTGTTTCTGCTTCT
FP110seq6-r	7824	TCCTTTGATCTTTTCTACGG
FP110seq7-f	7707	CAACATTTCCAAGAAAAGGA
FP110seq7-r	308	AGCACTGCACATAATGAATG
FP110seq8-r	576	GCTTACCCGTACTCAATGTT

2) Extraction and purification of plasmids

Recombinant pFP110 plasmid was transformed into *Corynebacterium glutamicum* ATCC13032. pFP110 plasmid DNAs were extracted from 2, 8 and 32

generations of culture of *C. glutamicum* APC199 by using QIAprep Spin Miniprep Kit (Cosmogenetech, Korea).

3) Sequence analysis

The sequences of pFP110 plasmid was verified by sequencing services provided by Cosmogenetech (Korea). Four foreign genes (*gmd*, *wcaG*, *α-1,2-ft*, and *lacY*) were identified using Multalin program (http://www-archbac.u-psud.fr/genomics/multalin.html).

3. Results

When the plasmid contig sequence was compared with pCN01, the positions of both primers used to insert four genes were identified. The vector backbone region outside these primers was 100% identical to the corresponding nucleotide sequences of pCN01. Also, multiple cloning site regions inside the primers were 100% identical to the nucleotide sequences of the four gene cassettes. The results confirmed that unintentional mutations of plasmid DNA did not occur during multiple generations of culture of *C. glutamicum* APC199. We concluded that the pFP110 plasmid can be maintained stable up to 32nd passages of culture.

Table C.2. Stability of plasmid pFP110 in multiple generations of culture

Passage	2nd Passage	8th Passage	32nd Passage
Sequence comparison with pFP110	Perfect match	Perfect match	Perfect match

Appendix D. Self-affirmed GMP Statement

Advanced Protein Technologies Corp.

Current Good Manufacturing Practices (cGMP) Statement

Food cGMP Statement of Advanced Protein Technologies Corp., Korea

Advanced Protein Technologies Corp. (herein after referred to as ‘APTech’), located in Paltan-myeon, Hwaseong-si, Gyeonggi-do, is currently building a plant meeting current Good Manufacturing Practices (cGMP). APTech will adhere to the principles of Hazard Analysis and Critical Control Point (HACCP) and cGMP to manufacture 2’ fucosyllactose and other human milk oligosaccharides. APTech is working with a certification firm to meet such standards.

These include Standard Operation Procedures to ensure and document that the materials used in commercial production are of a suitable grade to be used in food.

APTech is committed to providing innovative products that meet the expectations of our customers. This includes commitment to manufacture products safely and sustainably, and to meet applicable regulatory and statutory requirements through implementation of our quality and food safety management processes.

Sincerely,

Minseon Hong

Head of Quality Assurance

Advanced Protein Technologies, Corp.

Appendix E. Identification of *Corynebacterium glutamicum* APC199

1. Objective

To identify the test strain via *16S* rDNA sequencing and whole genomic sequencing.

2. 16S rDNA sequencing

Pure cultures of *Corynebacterium glutamicum* were grown on brain heart infusion (BHI) agar at 30°C for 24 hours. The plate was sent to Solgent Inc. (Daejun, South Korea) for bi-directional 16S rDNA sequencing. Bi-directional sequencing results were assembled using Codon Code Aligner (Codon Code Corporation, USA) and compared with the reference sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/Blastn/).

The results of sequencing 16s rDNA and analyzing it at BlastN are shown in Figure E.1. The colony taken from the cultured bacterium was found to have the same 16s rDNA sequence as the *C. glutamicum* ATCC strain when compared in BlastN.

Reference:
Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000),
"A greedy algorithm for aligning DNA sequences", J Comput Biol 2000;
7(1-2):203-14.

Database: 16S Microbial Sequences
20,470 sequences; 29,764,718 total letters

Query= sb1

Length=1370

Sequences producing significant alignments:		Score (Bits)	E Value
NR_041817.1	Corynebacterium glutamicum strain ATCC 13032 16S rib...	2519	0.0
NR_074663.1	Corynebacterium glutamicum strain ATCC 13032 16S rib...	2508	0.0

Figure E.1. The 16s rDNA sequencing result of *C. glutamicum.*

3. Whole genome sequencing of *Corynebacterium glutamicum* APC 199

DNA of *C. glutamicum* was extracted using MagListo Genomic DNA Extraction Kit (Qiagen). DNA was extracted after full growth of *C. glutamicum* and then the instructions of the manufacturer were followed. For whole genome sequencing of the extracted DNA, shearing was by AMPure XP magnetic beads with vortexing

(Theragenetex, Korea). The size and quality of the purified DNA was evaluated using Nanodrop and Bioanalyzer, and the quality assured DNA was annealed on SMRTbell templates (PacBio) and primers for analysis of whole genome sequencing using PacBio RS II system. The raw data were pre-assembled using SMRTpipe HGAP, while further assembling and polishing were performed using SMRTpipe Celera Assembler and SMRTpipe Quiver. Bioinformatics analysis including Genbank annotation was performed using RAST with matching the database of NCBI.

Whole genome sequencing results show the *C. glutamicum* test strain to contain one circular chromosomal DNA (Table E.2); it was taxonomically identified as *C. glutamicum* test strain according to the closest related neighborhood match. The comparative ANI value of the test strain and *C. glutamicum* ATCC13032 was calculated using whole genome sequence ANI calculating algorithm and showed a 99.99% match which proves a strong similarity between these two strains (Figure E.2, Tables E.1. and E.2). The similarities confirm that our test strain is not only a member strain of the species *C. glutamicum*, but it also has a close relationship with an ATCC standard strain.

Table E.1. Whole genome sequence overview of *C. glutamicum* test strain

Genome	*C. glutamicum* APC199
Taxonomy ID	6666666 (*Corynebacterium glutamicum*)
Domain	Bacteria
Taxonomy	Bacteria; Terrabacteria group; Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacterium; *Corynebacterium glutamicum*
Closest neighbor	*Corynebacterium glutamicum ATCC 13032*
Size (bp)	3,331,472
GC Content in the DNA	53.8 mol% G+C
Number of Contigs	1 circular (one chromosomal DNA)
Number of Coding Sequences	3224
Number of RNAs	78

Table E.2. Comparative genomic analysis of *C. glutamicum* test strain (A) and *C. glutamicum* ATCC13032 (B)

Metric	Value
ANI comparative value of A and B (%)	99.99 %
Genome A length (bp)	3,331,472
Genome B length (bp)	3,316,624

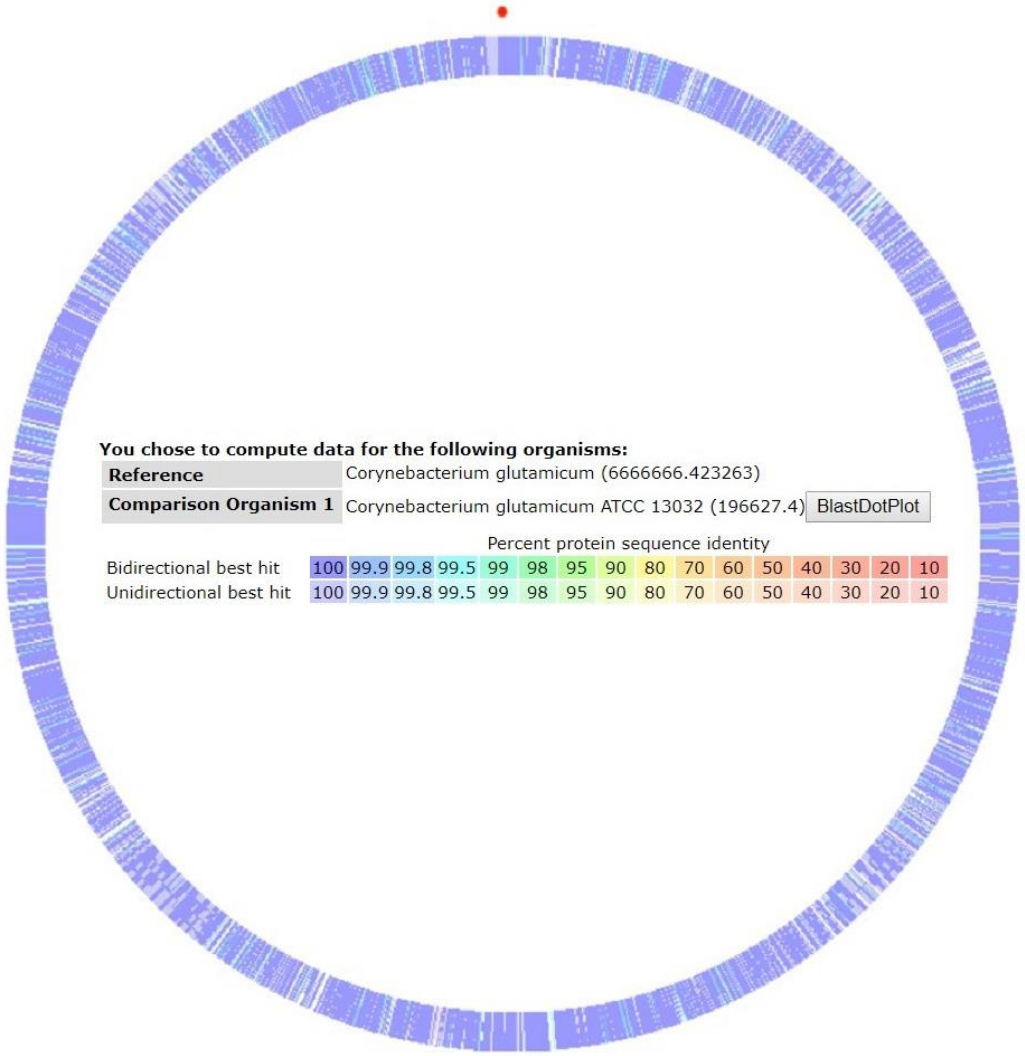

Figure E.2. Comparative genomic analysis of *C. glutamicum* test strain and *C. glutamicum* ATCC13032

Conclusions

The identity of the strain was confirmed as *Corynebacterium glutamicum* (whose strain name was designated as APC199) according to the National Center for Biotechnology Information (NCBI) database.

Appendix F. Certificates of Analysis

Appendix F.1. Methods of analysis are shown in Table F.1. The five non-consecutive samples, 2’-FL-CG-011 to 2’-FL-CG 015, were analyzed at third party Korean laboratories using Korean Ministry of Food and Drug Safety (KMFDS) methods of analysis. The Korean Ministry of Food and Drug Safety (KMFDS) methods of analysis and corresponding internationally recognized methods are listed in Table F.1.

For *Cronobacter sakazakii* and *E. coli*, all 5 samples initially analyzed at a Korean laboratory were re-analyzed at Eurofins using the sample sizes of 10 g and 25 g, respectively. Thus, the Korean methods of analysis for *Cronobacter sakazakii* and *E. coli* were not listed in Table F.1.

2’-FL and other carbohydrates (HPAEC-PAD), and protein (Bio-rad protein assay #5000006 based on Bradford assay) concentrations were analyzed in-house using validated methods. Endotoxin (Endosafe®-PTS™ [Version7.12B, Device 4486] cartridge type kit, Charles River) values were analyzed with Charles River’s FDA licensed endotoxin detection system. This system is harmonized with USP/EP guidelines and requirements (USP <1085>, EP <2.6.14>).

Table F.1. Methods of analysis

Parameters	Korean Ministry of Food and Drug Safety (KMFDS) methods	Equivalent internationally recognized method
Appearance (Color)	Visual	USP 34 Rev. <994>
Appearance (Form)	Visual	
Solubility in water	Visual	
Appearance in solution	Visual	
Water content, %	Karl Fischer titration	ASTM E203
Total ash, %	KMFDS No.2018-98, 8.2.1.2	AOAC 923.03
Arsenic, mg/kg	KS C IEC 62321-4 (2014), KS C IEC 62321-5 (2014), KS I ISO 17294:2014	IEC 62321-4 (2014), IEC 62321-5 (2014), ISO 17294:2014
Cadmium, mg/kg		
Lead, mg/kg		
Mercury, mg/kg		
Standard Plate Count, cfu/g	KMFDS No.2018-98, 8.4.5.1	AOAC 990.12
Yeast and Mold, cfu/g	KMFDS No.2018-98, 8.4.10	ISO 21527-2
Coliform, cfu/g	KMFDS No.2018-98, 8.4.7.2	AOAC 991.14
Staphylococcus aureus	KMFDS No.2018-98, 8.4.12.2	ISO 6888-1

Salmonella	KMFDS No.2018-98, 8.4.11	ISO 6579-1

Abbreviations: KMFDS = Korean Ministry of Food and Drug Safety; KS = Korean Industrial Standards; IEC = International Electrotechnical Commission; USP = US Pharmacopoeia; ASTM = The American Society for Testing and Materials; ISO = International Organization for Standardization; AOAC = Association of Official Analytical Chemists.

Table F.2 shows the Korean Ministry of Food and Drug Safety (KMFDS) methods of analysis and corresponding AOAC or ISO methods. Although the principle of each method is the same between KMFDS and AOAC/ISO methods, sample sizes may be different between KMFDS methods and corresponding AOAC or ISO methods.

Table F.2. Analysis method comparison

Contents	Korean method	Equivalent internationally recognized Method	Sample sizes in Korean methods
Ash	KMFDS No.2018-98, 8.2.1.2	AOAC 923.03	
Standard Plate Count	KMFDS No.2018-98, 8.4.5.1	AOAC 990.12	Sample size 25 g
Yeasts and Molds	KMFDS No.2018-98, 8.4.10	ISO 21527-2	Sample size 25 g
Coliform	KMFDS No.2018-98, 8.4.7.2	AOAC 991.14	Sample size 25 g
Staphylococcus aureus	KMFDS No.2018-98, 8.4.12.2	ISO 6888-1	Sample size 25 g
Salmonella	KMFDS No.2018-98, 8.4.11	ISO 6579-1	Sample size 25 g

Certificate of Test

KICET	Certificate No. : 2018-3855	KICET
388, Songnae-daero, Bucheon-si, Gyeonggi-do, Korea (Tel: +82 032 210 5110, Fax: +82 032 210 5115)	Page(1)/(1)Pages	

1. Client
   - ㅇ Company : Advanced Protein Technologies corp., / Chul-soo, Shin
   - ㅇ Address : 7th Floor Gyenggi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City, Gyeonggi-Province
   - ㅇ Date of Receipt : Nov. 23. 2018
2. Use of Report : Reference
3. Test Sample : 2FL-CG-011 Final
4. Date of Test : Nov. 23. 2018 ~ Dec. 13. 2018
5. Test method used : KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294(2014), Instrumental analysis
6. Testing Environment
   - ㅇ Temperature : (21 ± 3) ℃ , Humidity : (30 ± 5) % R.H.
7. Test Results

Sample name	Item	Results	Test Method	Remark
2FL-CG-011 Final	As (mg/kg) Pb (mg/kg) Cd (mg/kg) Hg (mg/kg)	< 0.001 < 0.001 < 0.001 < 0.001	KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294:2014, Instrumental analysis	

* used instrument : Perkin-elmer ELAN DRC II

Affirmation	Tested by Name : S. T. KIM	Technical Manager Name : O. S. AHN

Dec. 13. 2018

Korea Institute of Ceramic Engineering & Technology

양식번호 KT-QPF-26-04(03):2018.10.11 한국세라믹기술원

Certificate of Test

KICET	Certificate No. : 2018-4210-1	KICET
388, Songnae-daero, Bucheon-si, Gyeonggi-do, Korea (Tel: +82 032 210 5110, Fax: +82 032 210 5115)	Page(1)/(1)Pages	

1. Client
   - Company : Advanced Protein Technologies corp., / Chul-soo, Shin
   - Address : 7th Floor Gyenggi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City, Gyeonggi-Province
   - Date of Receipt : Dec. 19. 2018
2. Use of Report : Reference
3. Test Sample : 2FL-CG-012 Final
4. Date of Test : Dec. 19. 2018 ~ Jan. 09. 2019
5. Test method used : KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294(2014), Instrumental analysis
6. Testing Environment
   - Temperature : (17 ± 3) ℃ , Humidity : (35 ± 5) % R.H.
7. Test Results

Sample name	Item	Results	Test Method	Remark
2FL-CG-012 Final	As (mg/kg) Pb (mg/kg) Cd (mg/kg) Hg (mg/kg)	0.003 0.001 < 0.001 < 0.001	KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294:2014, Instrumental analysis	

* used instrument : Perkin-elmer ELAN DRC II

Affirmation	Tested by Name : S. T. KIM	Technical Manager Name : O. S. AHN

Jan. 09. 2019

Korea Institute of Ceramic Engineering & Technology

양식번호 KT-QPF-26-04[03]:2018.10.11 한국세라믹기술원

Certificate of Test

KICET
388, Songnae-daero, Bucheon-si, Gyeonggi-do, Korea
(Tel: +82 032 210 5110, Fax: +82 032 210 5115)

Certificate No. : 2018-4349
Page(1)/(1)Pages

1. Client
   - Company : Advanced Protein Technologies corp., / Chul-soo, Shin
   - Address : 7th Floor Gyenggi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City, Gyeonggi-Province
   - Date of Receipt : Dec. 28. 2018
2. Use of Report : Quality Control
3. Test Sample : 2FL-CG-013 Final
4. Date of Test : Dec. 28. 2018 ~ Jan. 16. 2019
5. Test method used : KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294(2014), Instrumental analysis
6. Testing Environment
   - Temperature : (17 ± 2) ℃ , Humidity : (35 ± 5) % R.H.
7. Test Results

Sample name	Item	Results	Test Method	Remark
2FL-CG-013 Final	As (mg/kg)	0.005	KS C IEC 62321-4(2014), KS C IEC 62321-5(2014), KS I ISO 17294:2014, Instrumental analysis	
	Pb (mg/kg)	0.005		
	Cd (mg/kg)	< 0.001		
	Hg (mg/kg)	< 0.001		

* used instrument : Perkin-elmer ELAN DRC II

Affirmation	Tested by Name : M. G. LEE	Technical Manager Name : O. S. AHN

Jan. 16. 2019

Korea Institute of Ceramic Engineering & Technology

Certificate of Test(Copy)

KICET	Certificate No. : 2019-1630-1	KICET
388, Songnae-daero, Bucheon-si, Gyeonggi-do, Korea (Tel: +82 032 210 5110, Fax: +82 032 210 5115)	Page(1)/(1)Pages	

1. Client
   - Company : Advanced Protein Technologies corp., / Chul-soo, Shin
   - Address : 7th Floor Gyenggi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-city, Gyeonggi-Province
   - Date of Receipt : Feb. 26. 2019
2. Use of Report : Quality control
3. Test Sample : 2FL-CG-014 Final
4. Date of Test : Feb. 26. 2019 ~ Mar. 06. 2019
5. Test method used : Wet analysis and instrumental analysis
6. Testing Environment
   - Temperature : (17 ± 2)℃, Humidity : (35 ± 5)% R.H.
7. Test Results

Sample name	Item	Results	Test Method	Remark
2FL-CG-014 Final	As (mg/kg)	ND	Wet analysis and instrumental analysis	LOQ <0.01mg/kg
	Pb (mg/kg)	ND		LOQ <0.01mg/kg
	Cd (mg/kg)	ND		LOQ <0.008mg/kg
	Hg (mg/kg)	ND		LOQ <0.01mg/kg

Ref. 1. ICP-MS Perkin-Elmer's ELAN DRC II
2. ND : Not Detected

Affirmation	Tested by Name : M. G. Lee	Tested by Name : W. S. Ahn

Mar. 06. 2019

Korea Institute of Ceramic Engineering & Technology

양식번호 KT-QPF-26-02(04):2018.10.11

Certificate of Test(Copy)

KICET	Certificate No. : 2019-1630-2	KICET
388, Songnae-daero, Bucheon-si, Gyeonggi-do, Korea (Tel: +82 032 210 5110, Fax: +82 032 210 5115)	Page(1)/(1)Pages	

1. Client
   - Company : Advanced Protein Technologies corp., / Chul-soo, Shin
   - Address : 7th Floor Gyenggi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-city, Gyeonggi-Province
   - Date of Receipt : Feb. 26. 2019
2. Use of Report : Quality control
3. Test Sample : 2FL-CG-015 Final
4. Date of Test : Feb. 26. 2019 ~ Mar. 06. 2019
5. Test method used : Wet analysis and instrumental analysis
6. Testing Environment
   - Temperature : (17 ± 2) ℃, Humidity : (35 ± 5) % R.H.
7. Test Results

Sample name	Item	Results	Test Method	Remark
2FL-CG-015 Final	As (mg/kg)	ND	Wet analysis and instrumental analysis	LOQ <0.01mg/kg
	Pb (mg/kg)	ND		LOQ <0.01mg/kg
	Cd (mg/kg)	ND		LOQ <0.008mg/kg
	Hg (mg/kg)	ND		LOQ <0.01mg/kg

Ref. 1. ICP-MS Perkin-Elmer's ELAN DRC II
2. ND : Not Detected

Affirmation	Tested by Name : M. G. Lee	Tested by Name : W. S. Ahn

Mar. 06. 2019

Korea Institute of Ceramic Engineering & Technology

양식번호 KT-QPF-26-02(04):2018.10.11

No. : D2019012300

Certificate of Analysis

Date of Application : 2019-01-28	Date of Manufacture :
No. of Sample : D2019012300	Expiration Date :
Lot No. :	
Inspection Purpose : Reference only	
Commodity : 2'-FL-CG-011	
Applicant — Name : Advanced Protein Technologies corp., Chul-soo, Shin	
Applicant — Company address : 7th Floor GyeongGi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City	

Analytical Result

Test Item	Result
Ash(%)	0.17 % (MFDS No.2018-98, 8.2.1.2)
Aflatoxin M_1 (μg/kg)	Not detected (MFDS No.2018-98, 8.9.2.3)
Standard plate count(/g)	0 (MFDS No.2018-98, 8.4.5.1.Dry rehydratable film method)
Mold & Yeast plate count(/g)	0 (MFDS No.2018-98, 8.4.10)
Coliform Group(/g)	0 (MFDS No.2018-98, 8.4.7.2.Dry rehydratable film method)
Escherichia coli(/g)	0 (MFDS No.2018-98, 8.4.8.2.Dry rehydratable film method)
Cronobacter spp.(/60g)	Negative (MFDS No.2018-98, 8.4.21)
Staphylococcus aureus(/g)	0 (MFDS No.2018-98, 8.4.12.2)
Salmonella spp.(/25g)	Negative (MFDS No.2018-98, 8.4.11)

Feb . 8 . 2019

We hereby certify that the above are correct.

Korea Health Supplements Association Sub. Korea Health Supplements Institute

Director : Yang, Joo-Hong

B-101, Korea Bio Park,, 700, Daewangpangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

KHSI

No. : D2019012301

Certificate of Analysis

Date of Application : 2019-01-28	Date of Manufacture :
No. of Sample : D2019012301	Expiration Date :
Lot No. :	
Inspection Purpose : Reference only	
Commodity : 2'-FL-CG-012	
Applicant — Name : Advanced Protein Technologies corp., Chul-soo, Shin	
Applicant — Company address : 7th Floor GyeongGi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City	

Analytical Result

Test Item	Result
Ash(%)	0.15 % (MFDS No.2018-98, 8.2.1.2)
Aflatoxin M_1 (μg/kg)	Not detected (MFDS No.2018-98, 8.9.2.3)
Standard plate count(/g)	0 (MFDS No.2018-98, 8.4.5.1.Dry rehydratable film method)
Mold & Yeast plate count(/g)	0 (MFDS No.2018-98, 8.4.10)
Coliform Group(/g)	0 (MFDS No.2018-98, 8.4.7.2.Dry rehydratable film method)
Escherichia coli(/g)	0 (MFDS No.2018-98, 8.4.8.2.Dry rehydratable film method)
Cronobacter spp.(/60g)	Negative (MFDS No.2018-98, 8.4.21)
Staphylococcus aureus(/g)	0 (MFDS No.2018-98, 8.4.12.2)
Salmonella spp.(/25g)	Negative (MFDS No.2018-98, 8.4.11)

Feb . 8 . 2019

We hereby certify that the above are correct.

Korea Health Supplements Association Sub. Korea Health Supplements Institute

Director : Yang, Joo-Hong

B-101, Korea Bio Park,, 700, Daewangpangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

KHSI

No. : D2019012302

Certificate of Analysis

Date of Application : 2019-01-28	Date of Manufacture :
No. of Sample : D2019012302	Expiration Date :
Lot No. :	
Inspection Purpose : Reference only	
Commodity : 2'-FL-CG-013	
Applicant — Name : Advanced Protein Technologies corp., Chul-soo, Shin	
Applicant — Company address : 7th Floor GyeongGi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City	

Analytical Result

Test Item	Result
Ash(%)	0.14 % (MFDS No.2018-98, 8.2.1.2)
Aflatoxin M_1 ($\mu g/kg$)	Not detected (MFDS No.2018-98, 8.9.2.3)
Standard plate count(/g)	0 (MFDS No.2018-98, 8.4.5.1.Dry rehydratable film method)
Mold & Yeast plate count(/g)	0 (MFDS No.2018-98, 8.4.10)
Coliform Group(/g)	0 (MFDS No.2018-98, 8.4.7.2.Dry rehydratable film method)
Escherichia coli(/g)	0 (MFDS No.2018-98, 8.4.8.2.Dry rehydratable film method)
Cronobacter spp.(/60g)	Negative (MFDS No.2018-98, 8.4.21)
Staphylococcus aureus(/g)	0 (MFDS No.2018-98, 8.4.12.2)
Salmonella spp.(/25g)	Negative (MFDS No.2018-98, 8.4.11)

Feb . 8 . 2019

We hereby certify that the above are correct.

Korea Health Supplements Association Sub. Korea Health Supplements Institute

Director : Yang, Joo-Hong

B-101, Korea Bio Park,, 700, Daewangpangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

KHSI

No. : D2019041998

Certificate of Analysis

Date of Application : 2019-04-18	Date of Manufacture :
No. of Sample : D2019041998	Expiration Date :
Lot No. :	
Inspection Purpose : Reference only	
Commodity : 2'-FL-CG-014	
Applicant — Name : Advanced Protein Technologies corp., Chul-soo, Shin	
Applicant — Company address : 7th Floor GyeongGi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City	

Analytical Result

Test Item	Result
Ash(%)	0.03 % (MFDS No.2018-98, 8.2.1.2)
Aflatoxin M_1 (μg/kg)	Not detected (MFDS No.2018-98, 8.9.2.3)
Standard plate count(/g)	0 (MFDS No.2018-98, 8.4.5.1.Dry rehydratable film method)
Mold & Yeast plate count(/g)	0 (MFDS No.2018-98, 8.4.10)
Coliform Group(/g)	0 (MFDS No.2018-98, 8.4.7.2.Dry rehydratable film method)
Escherichia coli(/g)	0 (MFDS No.2018-98, 8.4.8.2.Dry rehydratable film method)
Cronobacter spp.(/60g)	Negative (MFDS No.2018-98, 8.4.21)
Staphylococcus aureus(/g)	0 (MFDS No.2018-98, 8.4.12.2)
Salmonella spp.(/25g)	Negative (MFDS No.2018-98, 8.4.11)

Apr . 29 . 2019

We hereby certify that the above are correct.

Korea Health Supplements Association Sub. Korea Health Supplements Institute

Director : Yang, Joo-Hong

B-101, Korea Bio Park,, 700, Daewangpangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

KHSI

No. : D2019041999

Certificate of Analysis

Date of Application : 2019-04-18	Date of Manufacture :
No. of Sample : D2019041999	Expiration Date :
Lot No. :	
Inspection Purpose : Reference only	
Commodity : 2'-FL-CG-015	
Applicant — Name : Advanced Protein Technologies corp., Chul-soo, Shin	
Applicant — Company address : 7th Floor GyeongGi Bio-Center : 147 Gwanggyo-ro, Yeongtong-gu, Suwon-City	

Analytical Result

Test Item	Result
Ash(%)	0.09 % (MFDS No.2018-98, 8.2.1.2)
Aflatoxin M_1 (μg/kg)	Not detected (MFDS No.2018-98, 8.9.2.3)
Standard plate count(/g)	0 (MFDS No.2018-98, 8.4.5.1.Dry rehydratable film method)
Mold & Yeast plate count(/g)	0 (MFDS No.2018-98, 8.4.10)
Coliform Group(/g)	0 (MFDS No.2018-98, 8.4.7.2.Dry rehydratable film method)
Escherichia coli(/g)	0 (MFDS No.2018-98, 8.4.8.2.Dry rehydratable film method)
Cronobacter spp.(/60g)	Negative (MFDS No.2018-98, 8.4.21)
Staphylococcus aureus(/g)	0 (MFDS No.2018-98, 8.4.12.2)
Salmonella spp.(/25g)	Negative (MFDS No.2018-98, 8.4.11)

Apr . 29 . 2019

We hereby certify that the above are correct.

Korea Health Supplements Association Sub. Korea Health Supplements Institute

Director : Yang, Joo-Hong

B-101, Korea Bio Park,, 700, Daewangpangyo-ro, Bundang-gu, Seongnam-si, Gyeonggi-do, Republic of Korea

KHSI

eurofins | Food Integrity & Innovation

Report Number:	2752272-0
Report Date:	16-Jan-2020
Report Status:	Final

Certificate of Analysis

Advanced Protein Technologies Corp.
59-5 Seogeunnae-gil, Paltan-myeon
Hwaseong-si Gyeonggi-do 18575 Korea, Republic Of

Sample Name:	**2-FL CG011**	**Eurofins Sample:**	**9153159**
Project ID	ADVAN_PR_T-20200103-0001	**Receipt Date**	31-Dec-2019
PO Number	CVD	**Receipt Condition**	Ambient temperature
Sample Serving Size		**Login Date**	03-Jan-2020
		Date Started	03-Jan-2020
		Sampled	Sample results apply as received

Analysis	Result
E. coli	
Escherichia Coli	Absent /25 g
Enterobacter Sakazakii	
Enterobacter sakazakii	Absent /10 g

Method References	Testing Location
E. coli (USPE2022)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

USP Current revision, Chapter 2022.
To satisfy the requirements of the USP, the Preparatory Test must be completed on each matrix.
**Based on the results of the preparatory test, conditions stipulated are adequate for detecting the presence of the specified microorganism.

Enterobacter Sakazakii (SAKAISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA
Cronobacter sakazakii - ISO/TS 22964|IDF/RM 210:2006	

Testing Location(s)	Released on Behalf of Eurofins by
Food Integ. Innovation-Madison NE	**Shannon Jacoby - Business Unit Manager**

Eurofins Food Chemistry Testing US, Inc.
2102 Wright Street
Madison WI 53704
800-675-8375

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Report Number:	2752270-0
Report Date:	16-Jan-2020
Report Status:	Final

Certificate of Analysis

Advanced Protein Technologies Corp.
59-5 Seogeunnae-gil, Paltan-myeon
Hwaseong-si Gyeonggi-do 18575 Korea, Republic Of

Sample Name:	**2-FL CG012**	**Eurofins Sample:**	**9153157**
Project ID	ADVAN_PR_T-20200103-0001	**Receipt Date**	31-Dec-2019
PO Number	CVD	**Receipt Condition**	Ambient temperature
Sample Serving Size		**Login Date**	03-Jan-2020
		Date Started	03-Jan-2020
		Sampled	Sample results apply as received

Analysis	Result
E. coli	
Escherichia Coli	Absent /25 g
Enterobacter Sakazakii	
Enterobacter sakazakii	Absent /10 g
Preparatory Testing of Nutritional and Dietary Supplements	
E. coli Suitability Result	Pass**

Method References	Testing Location
E. coli (USPE2022)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

USP Current revision, Chapter 2022.
To satisfy the requirements of the USP, the Preparatory Test must be completed on each matrix.
**Based on the results of the preparatory test, conditions stipulated are adequate for detecting the presence of the specified microorganism.

Enterobacter Sakazakii (SAKAISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA
Cronobacter sakazakii - ISO/TS 22964|IDF/RM 210:2006	
Preparatory Testing of Nutritional and Dietary Supplements (USPE_PT)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

Testing Location(s)	Released on Behalf of Eurofins by
Food Integ. Innovation-Madison NE	**Shannon Jacoby - Business Unit Manager**

Eurofins Food Chemistry Testing US, Inc.
2102 Wright Street
Madison WI 53704
800-675-8375

eurofins | Food Integrity & Innovation

Report Number:	2752270-0
Report Date:	16-Jan-2020
Report Status:	Final

Certificate of Analysis

Advanced Protein Technologies Corp.
59-5 Seogeunnae-gil, Paltan-myeon
Hwaseong-si Gyeonggi-do 18575 Korea, Republic Of

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Report Number:	2752271-0
Report Date:	16-Jan-2020
Report Status:	Final

Certificate of Analysis

Advanced Protein Technologies Corp.
59-5 Seogeunnae-gil, Paltan-myeon
Hwaseong-si Gyeonggi-do 18575 Korea, Republic Of

Sample Name:	**2-FL CG013**	**Eurofins Sample:**	**9153158**
Project ID	ADVAN_PR_T-20200103-0001	**Receipt Date**	31-Dec-2019
PO Number	CVD	**Receipt Condition**	Ambient temperature
Sample Serving Size		**Login Date**	03-Jan-2020
		Date Started	03-Jan-2020
		Sampled	Sample results apply as received

Analysis	Result
E. coli	
Escherichia Coli	Absent /25 g
Enterobacter Sakazakii	
Enterobacter sakazakii	Absent /10 g

Method References	Testing Location
E. coli (USPE2022)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

USP Current revision, Chapter 2022.
To satisfy the requirements of the USP, the Preparatory Test must be completed on each matrix.
**Based on the results of the preparatory test, conditions stipulated are adequate for detecting the presence of the specified microorganism.

Enterobacter Sakazakii (SAKAISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA
Cronobacter sakazakii - ISO/TS 22964|IDF/RM 210:2006	

Testing Location(s)	Released on Behalf of Eurofins by
Food Integ. Innovation-Madison NE	**Shannon Jacoby - Business Unit Manager**

Eurofins Food Chemistry Testing US, Inc.
2102 Wright Street
Madison WI 53704
800-675-8375

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Report Number:	**2803359-0**
Report Date:	**03-Mar-2020**
Report Status:	**Final**

Certificate of Analysis

Advanced Protein Technologies Corp.

59-5 Seogeunnae-gil, Paltan-myeon
Hwaseong-si Gyeonggi-do 18575 Korea, Republic Of

Sample Name:	**2-FL CG014**	**Eurofins Sample:**	**9277749**
Project ID	ADVAN_PR_T-20200213-0002	**Receipt Date**	26-Feb-2020
PO Number	CVD	**Receipt Condition**	Ambient temperature
Sample Serving Size		**Login Date**	13-Feb-2020
		Date Started	26-Feb-2020
		Sampled	Sample results apply as received
		Online Order	15841-13087146

Analysis	Result
E. coli	
Escherichia Coli	Absent /25 g
Enterobacter Sakazakii	
Enterobacter sakazakii	Absent /10 g
Preparatory Testing of Nutritional and Dietary Supplements	
E. coli Suitability Result	Pass**

Method References	Testing Location
E. coli (USPE2022)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

USP Current revision, Chapter 2022.
To satisfy the requirements of the USP, the Preparatory Test must be completed on each matrix.
**Based on the results of the preparatory test, conditions stipulated are adequate for detecting the presence of the specified microorganism.

Enterobacter Sakazakii (SAKAISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA
Cronobacter sakazakii - ISO/TS 22964|IDF/RM 210:2006	
Preparatory Testing of Nutritional and Dietary Supplements (USPE_PT)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

Testing Location(s)	Released on Behalf of Eurofins by
Food Integ. Innovation-Madison NE	**Shannon Jacoby - Business Unit Manager**

Eurofins Food Chemistry Testing US, Inc.
2102 Wright Street
Madison WI 53704
800-675-8375

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Report Number:	**2803359-0**
Report Date:	**03-Mar-2020**
Report Status:	**Final**

Certificate of Analysis

Advanced Protein Technologies Corp.
59-5 Seogeunnae-gil, Paltan-myeon
Hwaseong-si Gyeonggi-do 18575 Korea, Republic Of

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Report Number:	**2803360-0**
Report Date:	**03-Mar-2020**
Report Status:	**Final**

Certificate of Analysis

Advanced Protein Technologies Corp.

59-5 Seogeunnae-gil, Paltan-myeon
Hwaseong-si Gyeonggi-do 18575 Korea, Republic Of

Sample Name:	**2-FL CG015**	**Eurofins Sample:**	**9277750**
Project ID	ADVAN_PR_T-20200213-0002	**Receipt Date**	26-Feb-2020
PO Number	CVD	**Receipt Condition**	Ambient temperature
Sample Serving Size		**Login Date**	13-Feb-2020
		Date Started	26-Feb-2020
		Sampled	Sample results apply as received
		Online Order	15841-13087146

Analysis	Result
E. coli	
Escherichia Coli	Absent /25 g
Enterobacter Sakazakii	
Enterobacter sakazakii	Absent /10 g
Preparatory Testing of Nutritional and Dietary Supplements	
E. coli Suitability Result	Pass**

Method References	Testing Location
E. coli (USPE2022)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

USP Current revision, Chapter 2022.
To satisfy the requirements of the USP, the Preparatory Test must be completed on each matrix.
**Based on the results of the preparatory test, conditions stipulated are adequate for detecting the presence of the specified microorganism.

Enterobacter Sakazakii (SAKAISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA
Cronobacter sakazakii - ISO/TS 22964|IDF/RM 210:2006	
Preparatory Testing of Nutritional and Dietary Supplements (USPE_PT)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

Testing Location(s)	Released on Behalf of Eurofins by
Food Integ. Innovation-Madison NE	**Shannon Jacoby - Business Unit Manager**

Eurofins Food Chemistry Testing US, Inc.
2102 Wright Street
Madison WI 53704
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Report Number:	**2803360-0**
Report Date:	**03-Mar-2020**
Report Status:	**Final**

Certificate of Analysis

Advanced Protein Technologies Corp.

59-5 Seogeunnae-gil, Paltan-myeon
Hwaseong-si Gyeonggi-do 18575 Korea, Republic Of

Advanced Protein Technologies Corporation

7th floor GyeongGi Bio-Center, 147 Gwanggyo-ro, Yeongtong-gu, Suwon-si,
Gyeonggi-do, 16229, Republic of Korea
Tel 82-31-888-6245, Fax 82-31-888-6247

Certificate of Analysis

PRODUCT: 2'-Fucosyllactose Powder
PRODUCT LOT: 2'-FL-CG-011
DATE OF MANUFACTURE: Oct. 29, 2018
DATE OF RELEASE: Feb. 19, 2019
STORAGE CONDITIONS: Room temperature

Analysis Results

Parameters	Specification	Results	Method
Protein content	≤ 100 μg/g	< 10	Bradford assay ; Bio-rad protein assay #5000006, APTech's validated internal method
2'-Fucosyllactose	≥ 94% (Area)	96.67	HPAEC-PAD, APTech's validated internal method
Lactose	≤ 5% (Area)	0.10	
3-Fucosyllactose	≤ 5% (Area)	ND	
Difucosyllactose	≤ 5% (Area)	0.24	
Fucosyl-Galactose	≤ 3% (Area)	ND	
Glucose	≤ 3% (Area)	1.13	
Galactose	≤ 3% (Area)	0.78	
Fucose	≤ 3% (Area)	ND	
Endotoxins	≤ 100 EU/g	< 7.2	Ph. Eur. 2.6.14; Endosafe®-PTS™ (Version7.12B, Device 4486) cartridge type kit (Charles River)

Limit of Quantitation : 2.6 ug/g, Bio-rad protein assay, based on the method of Bradford #5000006

Endosafe®-PTS™ cartridge sensitivity : 5 – 0.05 EU/mL, Endosafe®-PTS™ system is approved by FDA and harmonized with USP/EP guidelines and requirements. (USP <1085>, EP <2.6.14.>)

Quality Control Manager Ok-Seon Jeon

Advanced Protein Technologies Corporation

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Gyeonggi-do, 16229, Republic of Korea
Tel 82-31-888-6245, Fax 82-31-888-6247

Certificate of Analysis

PRODUCT: 2’-Fucosyllactose Powder
PRODUCT LOT: 2’-FL-CG-012
DATE OF MANUFACTURE: Dec. 10, 2018
DATE OF RELEASE: Feb. 19, 2019
STORAGE CONDITIONS: Room temperature

Analysis Results

Parameters	Specification	Results	Method
Protein content	≤ 100 μg/g	< 10	Bradford assay ; Bio-rad protein assay #5000006, APTech’s validated internal method
2’-Fucosyllactose	≥ 94% (Area)	95.93	HPAEC-PAD, APTech’s validated internal method
Lactose	≤ 5% (Area)	0.09	
3-Fucosyllactose	≤ 5% (Area)	ND	
Difucosyllactose	≤ 5% (Area)	0.86	
Fucosyl-Galactose	≤ 3% (Area)	ND	
Glucose	≤ 3% (Area)	1.28	
Galactose	≤ 3% (Area)	0.78	
Fucose	≤ 3% (Area)	ND	
Endotoxins	≤ 100 EU/g	< 5.7	Ph. Eur. 2.6.14; Endosafe®-PTS™ (Version7.12B, Device 4486) cartridge type kit (Charles River)

Limit of Quantitation : 2.6 ug/g, Bio-rad protein assay, based on the method of Bradford #5000006

Endosafe®-PTS™ cartridge sensitivity : 5 – 0.05 EU/mL, Endosafe®-PTS™ system is approved by FDA and harmonized with USP/EP guidelines and requirements. (USP <1085>, EP <2.6.14.>)

Quality Control Manager Ok-Seon Jeon _

Advanced Protein Technologies Corporation

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Gyeonggi-do, 16229, Republic of Korea
Tel 82-31-888-6245, Fax 82-31-888-6247

Certificate of Analysis

PRODUCT: 2’-Fucosyllactose Powder
PRODUCT LOT: 2’-FL-CG-013
DATE OF MANUFACTURE: Dec. 17, 2018
DATE OF RELEASE: Feb. 19, 2019
STORAGE CONDITIONS: Room temperature

Analysis Results

Parameters	Specification	Results	Method
Protein content	≤ 100 μg/g	< 10	Bradford assay ; Bio-rad protein assay #5000006, APTech’s validated internal method
2’-Fucosyllactose	≥ 94% (Area)	96.24	HPAEC-PAD, APTech’s validated internal method
Lactose	≤ 5% (Area)	0.10	
3-Fucosyllactose	≤ 5% (Area)	ND	
Difucosyllactose	≤ 5% (Area)	0.58	
Fucosyl-Galactose	≤ 3% (Area)	ND	
Glucose	≤ 3% (Area)	1.22	
Galactose	≤ 3% (Area)	0.78	
Fucose	≤ 3% (Area)	ND	
Endotoxins	≤ 100 EU/g	< 5	Ph. Eur. 2.6.14; Endosafe®-PTS™ (Version7.12B, Device 4486) cartridge type kit (Charles River)

Limit of Quantitation : 2.6 ug/g, Bio-rad protein assay, based on the method of Bradford #5000006

Endosafe®-PTS™ cartridge sensitivity : 5 – 0.05 EU/mL, Endosafe®-PTS™ system is approved by FDA and harmonized with USP/EP guidelines and requirements. (USP <1085>, EP <2.6.14.>)

Quality Control Manager Ok-Seon Jeon _ _

Advanced Protein Technologies Corporation

7th floor GyeongGi Bio-Center, 147 Gwanggyo-ro, Yeongtong-gu, Suwon-si,
Gyeonggi-do, 16229, Republic of Korea
Tel 82-31-888-6245, Fax 82-31-888-6247

Certificate of Analysis

PRODUCT: 2’-Fucosyllactose Powder
PRODUCT LOT: 2’-FL-CG-014
DATE OF MANUFACTURE: Dec. 20, 2018
DATE OF RELEASE: Jun. 05, 2019
STORAGE CONDITIONS: Room temperature

Analysis Results

Parameters	Specification	Results	Method
Protein content	≤ 100 μg/g	< 10	Bradford assay ; Bio-rad protein assay #5000006, APTech’s validated internal method
2’-Fucosyllactose	≥ 94% (Area)	96.84	HPAEC-PAD, APTech’s validated internal method
Lactose	≤ 5% (Area)	0.11	
3-Fucosyllactose	≤ 5% (Area)	ND	
Difucosyllactose	≤ 5% (Area)	0.02	
Fucosyl-Galactose	≤ 3% (Area)	ND	
Glucose	≤ 3% (Area)	1.00	
Galactose	≤ 3% (Area)	0.66	
Fucose	≤ 3% (Area)	ND	
Endotoxins	≤ 100 EU/g	< 5	Ph. Eur. 2.6.14; Endosafe®-PTS™ (Version7.12B, Device 4486) cartridge type kit (Charles River)

Limit of Quantitation : 2.6 ug/g, Bio-rad protein assay, based on the method of Bradford #5000006

Endosafe®-PTS™ cartridge sensitivity : 5 – 0.05 EU/mL, Endosafe®-PTS™ system is approved by FDA and harmonized with USP/EP guidelines and requirements. (USP <1085>, EP <2.6.14.>)

Quality Control Manager Ok-Seon Jeon

Advanced Protein Technologies Corporation

7th floor GyeongGi Bio-Center, 147 Gwanggyo-ro, Yeongtong-gu, Suwon-si,
Gyeonggi-do, 16229, Republic of Korea
Tel 82-31-888-6245, Fax 82-31-888-6247

Certificate of Analysis

PRODUCT: 2’-Fucosyllactose Powder
PRODUCT LOT: 2’-FL-CG-015
DATE OF MANUFACTURE: Jan. 02, 2019
DATE OF RELEASE: Jun. 05, 2019
STORAGE CONDITIONS: Room temperature

Analysis Results

Parameters	Specification	Results	Method
Protein content	≤ 100 μg/g	< 10	Bradford assay ; Bio-rad protein assay #5000006, APTech’s validated internal method
2’-Fucosyllactose	≥ 94% (Area)	97.99	HPAEC-PAD, APTech’s validated internal method
Lactose	≤ 5% (Area)	0.09	
3-Fucosyllactose	≤ 5% (Area)	ND	
Difucosyllactose	≤ 5% (Area)	0.02	
Fucosyl-Galactose	≤ 3% (Area)	ND	
Glucose	≤ 3% (Area)	0.63	
Galactose	≤ 3% (Area)	0.43	
Fucose	≤ 3% (Area)	ND	
Endotoxins	≤ 100 EU/g	35.5	Ph. Eur. 2.6.14; Endosafe®-PTS™ (Version7.12B, Device 4486) cartridge type kit (Charles River)

Limit of Quantitation : 2.6 ug/g, Bio-rad protein assay, based on the method of Bradford #5000006

Endosafe®-PTS™ cartridge sensitivity : 5 – 0.05 EU/mL, Endosafe®-PTS™ system is approved by FDA and harmonized with USP/EP guidelines and requirements. (USP <1085>, EP <2.6.14.>)

Quality Control Manager Ok-Seon Jeon

Appendix G. Quantitation of Residual DNA in 2’-FL by qPCR Technique

1. Objective

Quantitative polymerase chain reaction (qPCR) tests were conducted for 3 lots of 2’-FL powder to demonstrate that APTech’s 2’-FL preparations are free from contamination by host DNA and the four foreign genes, GDP-D-mannose-4,6-dehydratase(*gmd*), GDP-L-fucose synthase (*wcaG*), lactose permease (*lacY*), and α -1,2-fucosyltransferase (*α-1,2-ft*), introduced into pFP110 plasmid in *Corynebacterium glutamicum* APC199.

2. Materials and Methods

1) PCR primers and conditions

For the detection of *C. glutmaicum* APC199 originated genetic materials in purified 2’-FL samples, quantitative PCR (qPCR) was conducted by validated methods. qPCR tests were performed using specific primers designed by APTech.

Table G.1. Specific primers for detection of *C. glutamicum* host and four foreign genes

Primer name	Target	Sequence (5’ -> 3’)
16s rDNA Forward	*Corynebacterium glutamicum* ATCC13032 16s rDNA	ACCTGGAGAAGAAGCACCG
16s rDNA Reverse		TCAAGTTATGCCCGTATCG
gmd Forward	pFP110 (*Escherichia coli* ATCC 700926)	CTGTTGACCCGCGTTACTT
gmd Reverse		TGAGGGTGATTTCCGGTTTC
wcaG Forward	pFP110 (*Escherichia coli* ATCC 700926)	ATGAAGTCTGGCTGGAGAAC
wcaG Reverse		CCACTTTGGCGATGGTTTG
α-1,2-ft Forward	pFP110 *(Pseudopedobacter saltans* ATCC 51119)	CTGCCGAAGATCTTTACCTTCT
α-1,2-ft Reverse		ATAAAGGGCGGTCTTCGTAAA
lacY Forward	pFP110 *(E. coli* ATCC 700926)	TGCCGCTATTTCTCTGTTCTC-
lacY Reverse		CGGTAATAATCCACAGCAGGTAT

Abbreviations: *α-1,2-ft* =α-1,2-fucosyltransferase; *gmd*= GDP-D-mannose-4,6-dehydratase; *wcaG*=GDP-L-fucose synthase; *lacY*=lactose permease

The target genes were amplified using 2x SYBR Green master mix as supplied by CELLSAFE. Dnase/RNase free water 13 ul and 2xSYBR Green master mix 20 ul was pipetted into a 96 well plate. Forward and Reverse primers (1 pmole) 2 ul each was pipetted into a 96 well plate. 5 ul template DNA or distilled water was added into the 96 well plate. Total PCR reaction mixture was 40 ul in each well. The plate was placed in the QuantStudio 3 Real-Time PCR (ThermoFisher Scientific, USA) and appropriate program was chosen for the amplification of sample mixture.

qPCR reaction was done in a typical method, starting at an initial denaturation temperature at 95°C for 10 min, 35 cycles of 15 sec denaturation at 95°C, 15 sec annealing at 59°C and 30 sec elongation at 72°C. After the last cycle, an additional 5 min elongation step at 72°C was performed.

2) Limit of detection level of qPCR

To determine the limit of detection level of *C. glutamicum* APC199 genetic materials in the final 2'-FL powder, pFP110 plasmid DNA and APC199 genomic DNA were prepared from *C. glutamicum* APC199 using plasmid mini kit (QIAGEN, Germany) and LaboPass^{TM} Bacteria mini (Cosmogenetech, Korea). The quantitation of purified DNA was performed by Epoch^{TM} Multi Volume Spectrophotometer system (BioTek, USA).

Template DNA was used to dilute to a concentration of 10 ng/ul. pFP110 plasmid DNA and APC 199 genomic DNA were diluted to a concentration of 1 ng/ul using DNase/RNase free water. Eight independent 10-fold series of dilutions (10 ng/ul to 1 x 10^{-7} ng/ul) were prepared. Tests were done in duplicate and in three runs. The qPCR reactions were performed in the same method as described above. After qPCR reaction, each limit of detection level was decided by Ct (threshold cycle) value. When the Ct value is less than 30, we conclude the detection of qPCR product as positive. When the qPCR products were undetermined or Ct value was greater than 30, we regarded the results as negative.

3) Detection of host genomic DNA and four foreign genes from 2'-FL powder

qPCR tests were conducted for 3 independent batches of purified 2'-FL powder (2'-FL-CG-013, 2'-FL-CG-014, 2'-FL-CG-015). We used DNase/RNase free water as a negative control, pFP110 DNA and APC199 genome DNA of 1 ng/ul concentration as a positive control.

Each 2’-FL sample was prepared as 500 g/L solution using DNase/RNase-free water. The qPCR reactions were performed using the same method as the procedure for determining the LOD value.

3. Results

1) LOD (Limit of detection) of qPCR

qPCR reactions were performed by serial dilution of the DNA concentration of pFP110 plasmid DNA and APC199 genomic DNA from 1 ng/ul to 1 x 10^{-7} ng/ul. qPCR reactions were done in duplicate and three runs for each 2’-FL sample. The PCR product of *gmd*, *wcaG*, *α-1,2-ft*, and *lacY* genes in pFP110 were amplified at 10^{-5} ng/ul, but not at 10^{-6} ng/ul concentration.

16s DNA of *C. glutamicum* APC199 genome was amplified at 10^{-3} ng/ul, but not at 10^{-4} ng/ul concentration.

The results show that detection limit of *gmd*, *wcaG*, *α-1,2-ft*, and *lacY* was less than 6.25x10^{-6} ng/ul (6.25x10^{-3} ppb) and 16s DNA in APC199 genome was less than 1.25x10^{-3} ng/ul (1.25 ppb).

2) Detection of host genomic DNA and four foreign genes in 2’-FL powder

From the above results, limits of detection were determined to be less than 6.25x10^{-3} ppb for *gmd*, *wcaG*, *α-1,2-ft*, and *lacY* and 1.25 ppb for 16s DNA. To detect residual DNA from the 2’-FL powder manufactured from *C. glutamicum* APC199, the three independent batches of 2’-FL (2’-FL-CG-013, 2’-FL-CG-014, 2’-FL-CG-015) were prepared in a 500 g/L solution and tested, 1 ng/ul of pFP110 plasmid DNA and APC199 genomic DNA was used as a positive control. qPCR reactions were conducted in duplicate and three runs. DNA amplicons were not detected in all samples after qPCR reactions and the results show that 2’-FL manufactured by APTech were free of detectable DNA from host genome and four foreign genes in pFP110 plasmid of *C. glutamicum* APC199.

4. Conclusion

It was concluded that no residual genes are present in the finished 2’-FL ingredients.

Table G.2. Evaluation of 2’-FL for absence of residual DNA of the *C. glutamicum* APC199

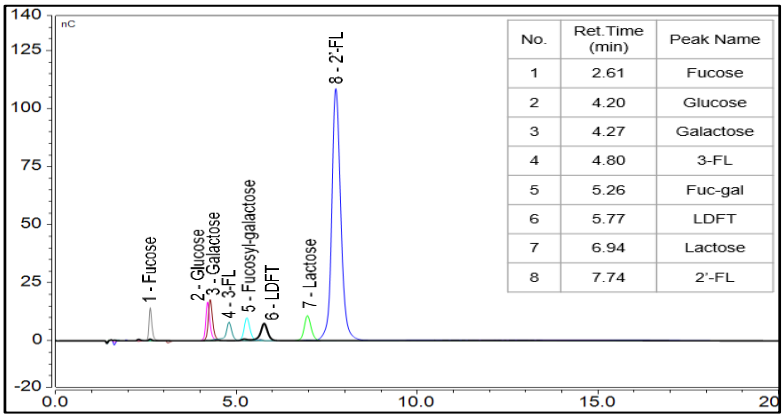
Target gene	LOD	2’-FL samples		
		2’-FL-CG-013	2’-FL-CG-014	2’-FL-CG-015
16s DNA	1.25×10^{-3} ng/ul	< LOD	< LOD	< LOD
gmd	6.25×10^{-6} ng/ul	< LOD	< LOD	< LOD
wcaG	6.25×10^{-6} ng/ul	< LOD	< LOD	< LOD
α-1,2-ft	6.25×10^{-6} ng/ul	< LOD	< LOD	< LOD
lacY	6.25×10^{-6} ng/ul	< LOD	< LOD	< LOD
Note : Production strain *C. glutamicum* APC199 with pFP110 plasmid LOD = limit of detection for the individual genes; ng = nanogram; ul= microliter; qPCR = quantitative polymerase chain reaction				

Appendix H. Chemical Identification and Analysis of 2’-FL

1. HPAEC-PAD

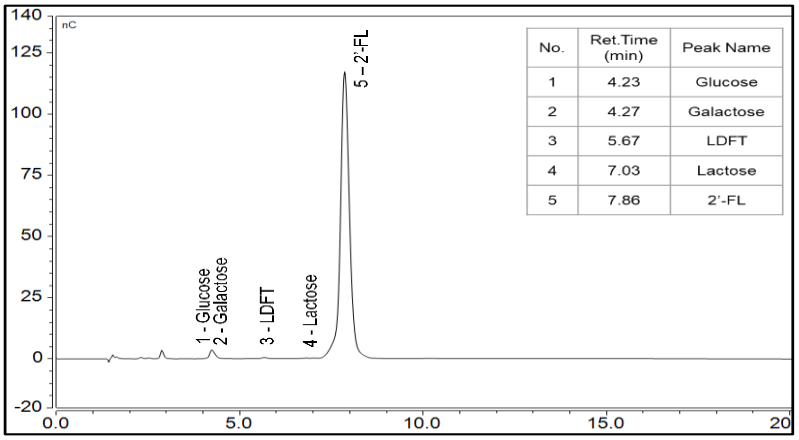
APTech’s 2’-FL was analyzed by HPAEC-PAD (high performance anion exchange chromatography with pulsed amperometric detection). Standards of 2’-FL, LDFT, 3-FL and fucosyl-galactose were purchased from Carbosynth Limited, UK (Carbosynth). Galactose and fucose were purchased from Sigma-Aldrich, USA. Lactose and glucose were from Duksan Pure Chemicals Co. Ltd, Korea. Samples were analyzed in the ICS 5000 system (Dionex) equipped with the Carbopac PA-100 column (250 x 4mm, Dionex). The eluents consisted of (A)100 mM NaOH and (B)100 mM NaOH containing 300 mM sodium acetate. The elution conditions were 0.3% (B) buffer for 20 min for analysis, and then 25% (B) buffer for 10 min for column washing followed by 0.3% (B) buffer for 15 min to stabilize the column. APTech's 2’-FL showed similar retention time as the reference material, and the analysis showed a purity of > 94%.

(a)



No.	Ret.Time (min)	Peak Name
1	2.61	Fucose
2	4.20	Glucose
3	4.27	Galactose
4	4.80	3-FL
5	5.26	Fuc-gal
6	5.77	LDFT
7	6.94	Lactose
8	7.74	2'-FL

(b)



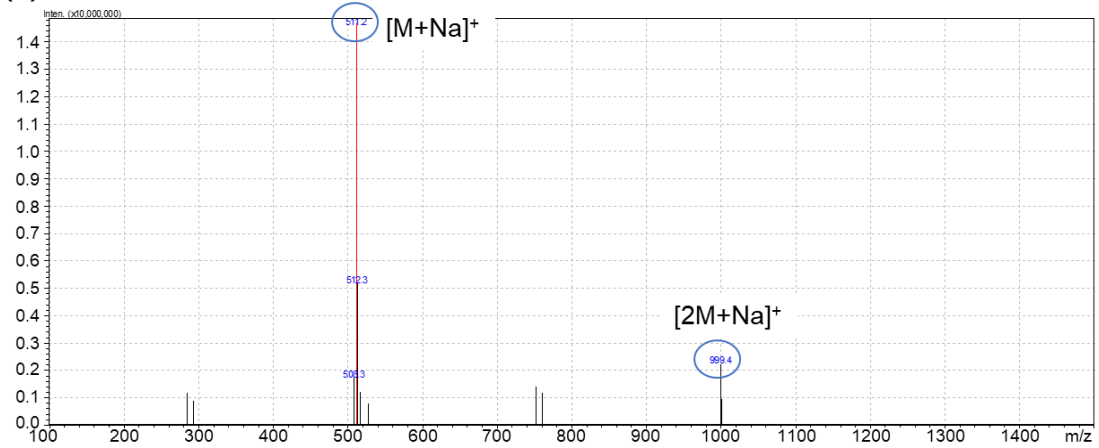
No.	Ret.Time (min)	Peak Name
1	4.23	Glucose
2	4.27	Galactose
3	5.67	LDFT
4	7.03	Lactose
5	7.86	2'-FL

Figure H.1. HPAEC-PAD chromatogram of 2’-FL. (a) 8 standard chromatograms (b) APTech’s 2’-FL chromatogram.

2. Mass Spectrometric Analysis of 2'-FL: Comparison of APTech 2'-FL with Reference Material (Carbosynth)

2'-FL powder manufactured by APTech was compared with the reference material (Carbosynth) using liquid chromatography tandem mass spectrometry (Figure 2). The samples were analyzed using the high sensitivity triple quadrupole mass spectrometer LCMS-8050 (Shimadzu, Japan) coupled to a NEXERA X2 LC (Shimadzu, Japan) system. The LCMS was operating in ESI-positive mode. The molecular weight of 2'-FL is 488.4 (Molecular Formula : $C_{18}H_{32}O_{15}$). Identical positive fragmentation patterns were observed when comparing the main component of the APTech's 2'-FL and 2'-FL reference (Carbosynth). The main peak of APTech's 2'-FL powder was 511.2 mass-to-charge ratio (m/z) for $[M+Na]^+$ in positive ion mode, identical to the reference material (Carbosynth).

(a)



(b)

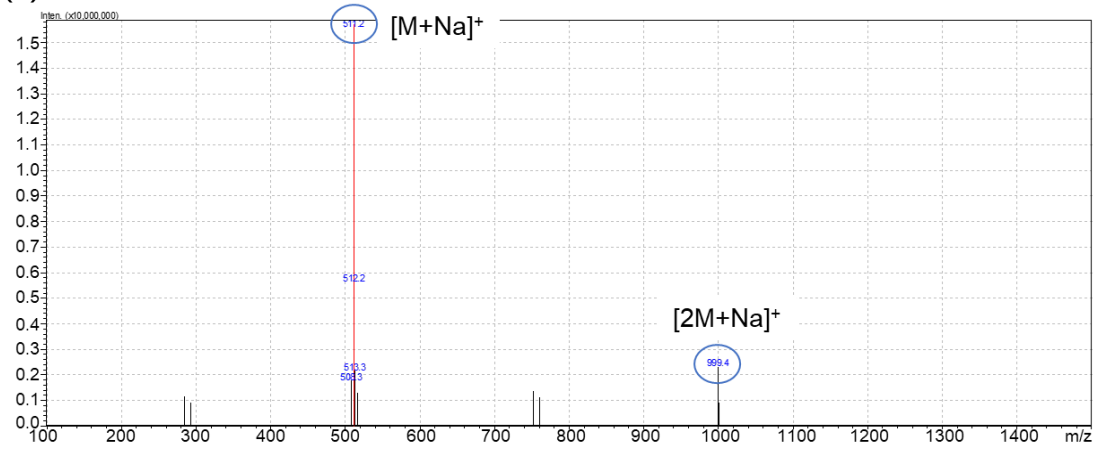


Figure H.2. Mass spectra of 2'-FL showing in positive ionization mode. (a) 2'-FL of Carbosynth (b) 2'-FL of APTech.

Imprint	Director	Head of cooperation
		

HANKUK UNIVERSITY OF FOREIGN STUDIES
Research Affairs/R&DB Foundation

NMR data Report

Project	**Conformation of the structure of 2’-FL using Solution-state NMR**	
Period	**14th Aug, 2019 ~ 22nd Aug, 2019**	
Analytical institution	**Directed by**	**Yongae Kim**
	Tested by	**Ji-Ho Jeong**
		Minseon Kim
Client	**Company**	**AP Technologies Corp.**
	Requester	**Chang-Ku Jeong**
		Ok-Seon Jeon

1. Summary

This experiment was carried out using NMR technique to investigate the structural similarity of three 2’-FL ingredients (provided by Carbosynth, IsoSep, and AP Tech).

2. Materials and reagents

‘Carbosynth, IsoSep, and AP Tech’ samples provided by AP Technologies were used without any pretreatment. Solvent (D_2O) was used for NMR measurement.

3. Sample pretreatment for NMR experiment

Each 8 mg sample taken from ‘Carbosynth, IsoSep, and AP Tech’ was dissolved in 400 µl of D_2O and transferred to a 5 mm NMR tube.

4. Experimental procedure

To obtain high resolution NMR spectra, Bruker Avance III HD 400 MHz NMR spectrometer at 9.4 Tesla (Bruker Biospin, Rheinstetten, Germany) was used to perform water suppression ^{1}H NMR and ^{13}C NMR experiment. ^{1}H NMR spectra were run using the typical Bruker pulse sequence *zgpr* (1D water presaturation). The scan number (NS) was set to 16, acquisition time (AQ) to 3 seconds and the spectral width (SW) to 8 ppm. ^{13}C NMR spectra were acquired by using the Bruker pulse sequence *zgpg* 30. The NS was set to 10240, AQ to 1.5 seconds and SW to 296 ppm. All 1D NMR spectra were obtained using Bruker Topspin 3.5 software.

5. Results

Comparative analysis of ^{1}H NMR spectra of Carbosynth, IsoSep, and APTech samples

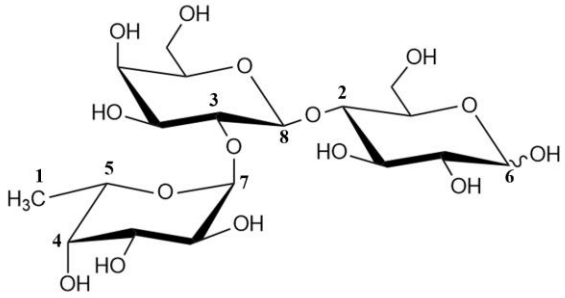


Figure H.3. Expected structure of test compound

Table H.1. Assignment of ^{1}H NMR Chemical Shifts

^{1}H Chemical shift table	
Number	**^{1}H Chemical shift, δ**
1	**1.1**
2	**3.2**
3	**3.4**
4	**3.5**
5	**4.1**
6	**4.4, 4.5**
7	**5.1**
8	**5.2**

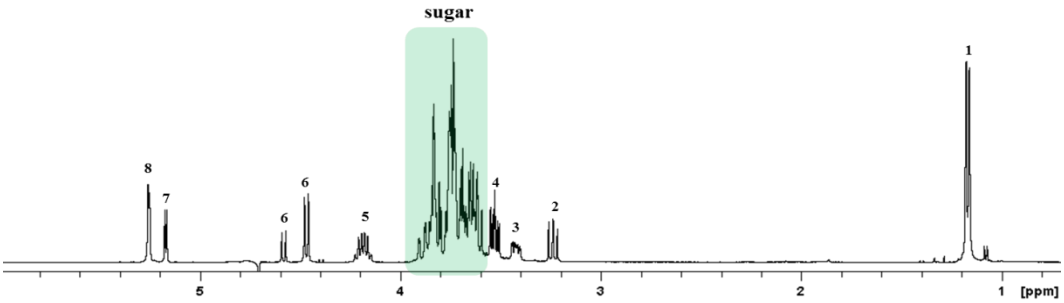


Figure H.4. ^{1}H NMR spectrum of AP Tech

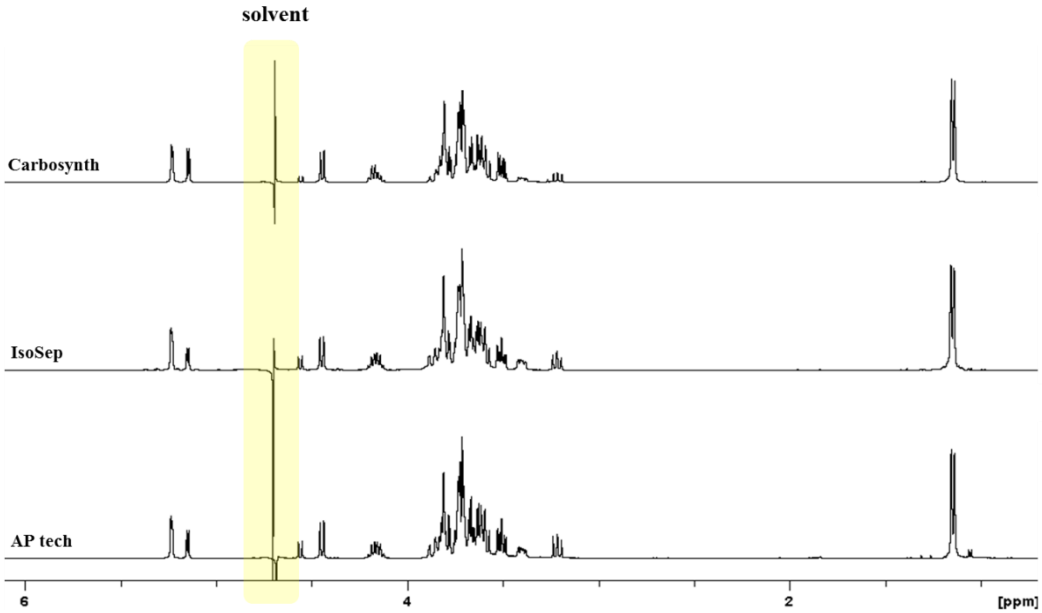


Figure H.5. Comparative analysis of ^{1}H NMR spectra of Carbosynth, IsoSep, and APTech

Comparative analysis of ^{13}C NMR spectra of Carbosynth, IsoSep, AP tech

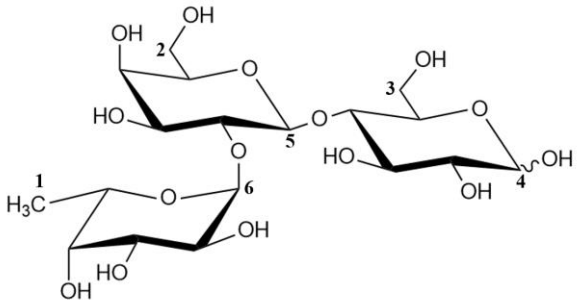


Figure H.6. Expected structure of test compound

Table H.2. Assignment of ^{13}C NMR Chemical Shifts

^{13}C Chemical shift table	
Number	**^{13}C Chemical shift, δ**
1	**15.2**
2	**60.3**
3	**61.3**
4	**91.8, 95.9**
5	**99.4**
6	**100.3**

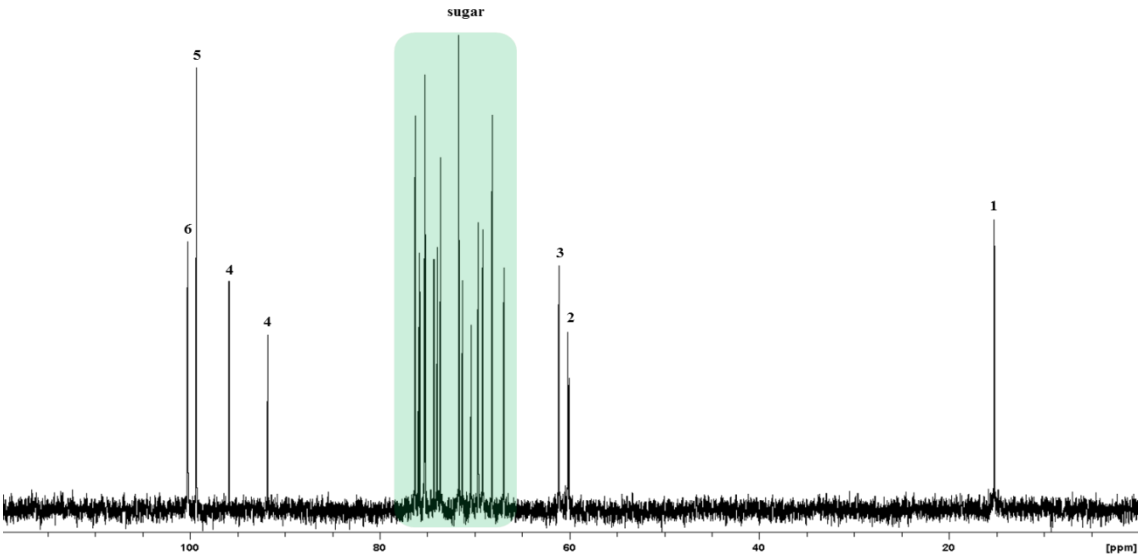


Figure H.7. ^{13}C NMR spectrum of APTech

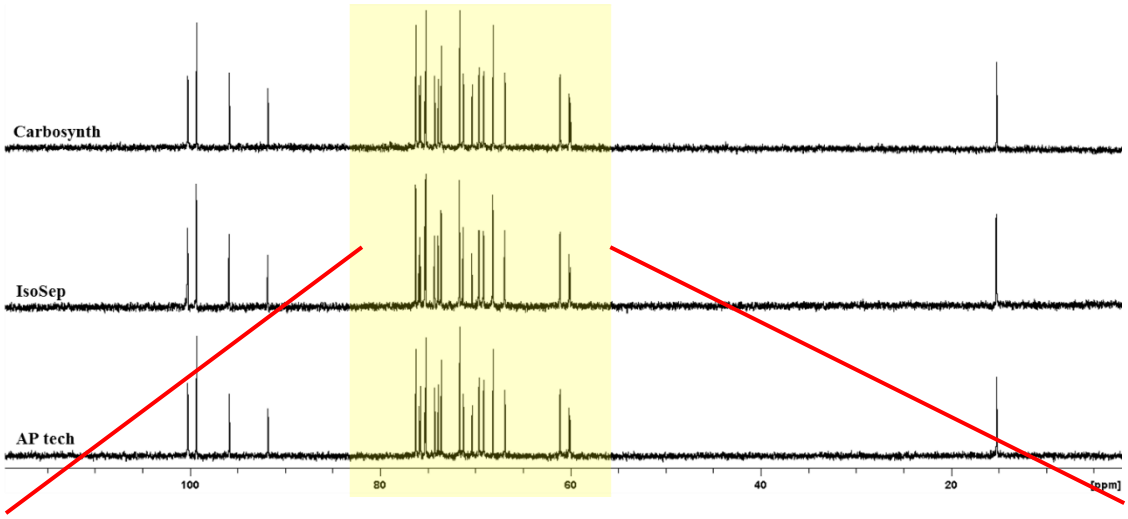


Figure H.8. Comparative analysis of ^{13}C NMR spectra of Carbosynth, IsoSep, and APTech

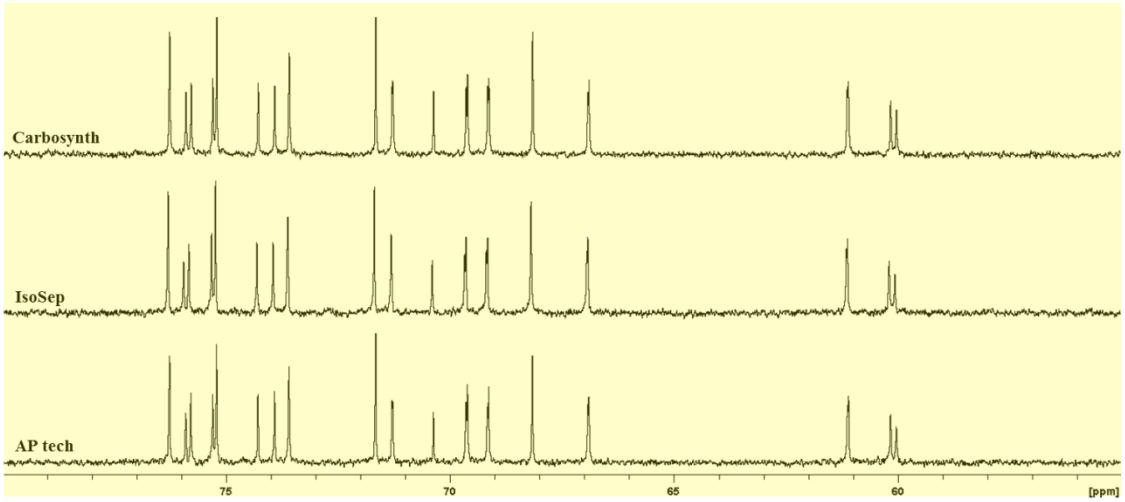


Figure H.9. Inset spectra of the corresponding to 2-FL in Figure H.8.

6. Conclusion

- As a result of ^{1}H water suppression NMR experiment on three kinds of sugar compound samples (Carbosynth, IsoSep, AP tech), all peaks except the ^{1}H peak from solvent were found to be the same.

- The ^{13}C NMR peaks appearing in all regions of the spectrum appeared to be the same for all three samples (Carbosynth, IsoSep, and APTech).

- Because the complex ^{13}C peaks observed at 55 ppm to 80 ppm appeared at the same position and the splitting of the peaks due to coupling was the same, all three samples are thought to have the same molecular structure.

- Combining the above data, test substances provided by Carbosynth, IsoSep, and APTech are concluded to have the identical structure, 2’-fucosyllactose.

Appendix I. 2’-FL Content in Human Milk

Location	Days or months after postpartum	2’-FL content (g/L)	References
Ethiopia – Rural	71 d	1.11	McGuire et al., 2017
Ethiopia – Urban	59 d	1.39	
Gambia – Rural	65 d	1.44	
Gambia - Urban	62 d	2.06	
Ghana	58 d	0.70	
Kenya	73 d	1.65	
Peru	60 d	3.19	
Spain	70 d	1.91	
Sweden	49 d	2.77	
Washington, USA	68 d	2.03	
California, USA	62 d	3.44	
China - Urban	5-11 d	2.00	Austin et al., 2016
	12-30 d	1.90	
	1-2 mo	1.70	
	2-4 mo	1.30	
	4-8 mo	1.10	
Not specified	Not specified	2.7	Donovan and Comstock, 2016
Not specified	14 d	2.87	Goehring et al., 2014
Samoa	5-10 d	0.22	Leo et al., 2010
	22-155 d	0.69	
Japan	1 d	2.49	Asakuma et al., 2008
	2 d	2.01	
	3 d	1.58	
Not specified	1 wk of lactation	4.53-6.27	Balogh et al., 2015
		2.69-3.55	
Asia	Not specified	2.1	Castanys-Munoz et al., 2013
Europe		2.6	
Latin America		2.48	
USA		2.0	
Mexico City	30-60 d	1.21	Chaturvedi et al., 1997
Not specified	1-3 d	0.24-0.36	Grollman and Ginsburg, 1967
	5 wk	0.46	
	6 wk	0.031	
California, USA	Not specified	2.40-3.70	Marx et al., 2014
Japan	4 d	0.20	

	10 d	0.34	Sumiyoshi et al., 2003
	30 d	0.29	
	100 d	0.05	
Burkinabe, Africa	1 d	1.80	Musumeci et al., 2006
	2 d	4.50	
	3 d	8.40	
Italy	1 d	1.00	
	2 d	2.10	
	3 d	4.20	
Samoa	5-10 d	0.22	Leo et al., 2009
	>10 d	0.69	
Italy	4 d	7.3	Gabrielli et al., 2011
	5-10 d	6.05	
	>10 d	5.25	
US	1 d	2.8	Chaturvedi et al., 2001a
	2 d	3	
	3 d	3.5	
	>10 d	3.6	
Asia	0-2 d	2.29	Erney et al., 2000
	3-10 d	2.26	
	11-30 d	2.36	
	>31 d	1.50	
Europe	0-2 d	3.40	
	3-10 d	2.69	
	11-30 d	2.38	
	>31 d	2.36	
Latin-America	3-10 d	2.79	
	11-30 d	2.61	
	>31 d	1.91	
US	3-10 d	2.78	
	11-30 d	2.56	
	>31 d	1.69	
Mixed geographies		2.4	
	2 d	2.8	
	4 d	2.6	
	>10 d	2.25	
Germany	2-28 d	0.45	Kunz et al., 1999
Europe	4 d	3.93	Coppa et al., 1999
	10 d	3.02	
	30 d	2.78	
	60 d	1.84	
	90 d	2.46	

Germany	5-10 d	3.37	Thurl et al., 2010
	>10 d	2.96	
Europe	Mature milk	1.84	Thurl et al., 1996
US	0-33 d	1.13	Nahkla et al., 1999
	4-128 d	1.27	
America and Europe	1-100 d	2.38	Erney et al., 2001
Latin America	1-100 d	3.95	Morrow et al., 2004
Japan	30-120 d	1.48	Asakuma et al., 2011
Europe	25-35 d	0-2.66	Coppa et al., 2011
Europe	4-30 d	0-7.15	Galeotti et al., 2012
US	3 d	1.12	Bao et al., 2013
	14-29 d	1.08	
US	90 d	1.22	Smilowitz et al., 2013
Europe	4-30 d	0-7.80	Galeotti et al., 2014
US	35 d	0.48-2.50	Hong et al., 2014

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Appendix J. Toxicological Evaluation of 2'- Fucosyllactose (2'-FL) in Rats

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[1]NutraSource, Inc.; [2]Advanced Protein Technologies Corporation (APTech)

The summary was prepared based on the reports issued by Biotoxtech, a Good Laboratory Practice (GLP) certified lab, in South Korea.
Biotoxtech is located at 53, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongju-si, Chungcheongbuk-do, 28115, Republic of Korea

The basis of this summary is as follows:

1) Biotoxtech. 2019a. Bacterial Reverse Mutation Test of 2'-Fucosyllactose. Study Number B18674. Study Director, Seung-Young Hong
2) Biotoxtech. 2019b. *In Vitro* Mammalian Chromosomal Aberration Test of 2'-Fucosyllactose. Study Number B18675. Study Director, Seung-Young Hong
3) Biotoxtech. 2019c. *In vivo* Micronucleus Test of 2'-Fucosyllactose in ICR mice. Study Number B18676. Study Director, Seung-Young Hong
4) Biotoxtech. 2019d. Single Oral Dose Toxicity Study of 2'-Fucosyllactose in Juvenile Sparague-Dawley rats. Study Number B18672. Study director, Chung-Tack Han
5) Biotoxtech. 2019e. Ninety-Day Repeated Oral Dose Toxicity Study with a Four Week Recovery Period of 2'-Fucosyllactose in Juvenile Sparague-Dawley rats. Study Number B18673. Study Director, Chung-Tack Han

ABSTRACT

The safety of 2'-fucosyllactose (2'-FL, purity ≥94%) produced by fermentation via genetically engineered *Corynebacterium glutamicum* APC199 was evaluated in acute and subchronic toxicity studies as well as a battery of mutagenicity and genotoxicity studies. The *in vitro* mutagenicity study using reverse bacterial mutation tests and the *in vitro* genotoxicity study with Chinese hamster lung cells demonstrated that 2'-FL was not mutagenic or clastogenic in the presence or absence of metabolic activation. In the *in vivo* mouse micronucleus assay, 2'-FL did not induce micronuclei formation in the bone marrow cells of mice, indicating that it is non-clastogenic. The acute toxicity study found that the approximate lethal dose of 2'-FL was much greater than 7,500 mg/kg, the highest dose tested, in male and female juvenile rats (7 days old). In the 90-day oral toxicity study, the no observed adverse effect level (NOAEL) of 2'-FL was determined to be 7,500 mg/kg bw/day, the highest dose tested, in male and female rats. The results of these studies support the safety of 2'-FL as a food ingredient.

DETAILS

Bacterial Reverse Mutation Test of APTech’s 2’-FL

The potential mutagenicity of APTech’s 2’-FL (purity, ≥94%) was evaluated in histidine-requiring *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) strains and a tryptophan-requiring *Escherichia coli* (WP2*uvrA*(pKM101) strain in the presence or absence of metabolic activation (S9). In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was selected at 5,000 µg/plate, and it was sequentially diluted by applying a geometric ratio of 4 to produce 5 lower dose levels (1,250, 313, 78.1, 19.5, and 4.88 µg/plate). As a result, growth inhibition and precipitation of the test substance were not evident at any dose level in all strains in the presence and absence of the metabolic activation.

In the main study, the bacterial strains were treated with 2’-FL at concentrations of 0, 313, 625, 1,250, 2,500, and 5,000 µg/plate. Also, the negative and positive control groups (2-nitrofluorene for TA98, sodium azide for TA100 and TA1535, 9-aminoacridine for TA1537, and 4-nitroquinoline N-oxide for WP2*uvrA* (pKM101) in the absence of metabolic activation; and 2-aminoanthracene for all strains in the presence of metabolic activation) were used in both experiments. The growth inhibition and deposition of the test substance were not evident at any dose levels of all strains in the absence and presence of metabolic activation (Table J.1). In the positive control group, the mean number of revertant colonies was markedly increased more than twice when compared to the negative control group. Thus, 2’-FL was determined to be non-mutagenic in the Ames test at concentrations up to 5,000 µg/plate under the test conditions.

In Vitro Chromosome Aberration Test of APTech’s 2’-FL

This study was designed to evaluate the potential of 2’-FL (purity, ≥94%) to induce chromosomal aberrations in Chinese Hamster Lung (CHL/IU) cells). To evaluate the ability of 2’-FL to induce chromosomal aberrations in cultured CHL/IU cells with and without S9 metabolic activation, two separate *in vitro* chromosome aberration assays were conducted. Dimethyl sulphoxide (DMSO) served as both the diluent for 2’-FL and the negative control substance. Mitomycin C and benzo[a]pyrene were used for the positive controls in the absence or presence of S9 metabolic activation, respectively. In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was selected at 5,000 µg/mL, and it was sequentially diluted by applying a geometric ratio of 2 to produce lower dose levels (2,500, 1,250, 625, 313, 156, 78.1, 39.1, and 19.5 µg/mL). As a result, cytotoxicity and precipitation of the test substance were not evident in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation.

Therefore, the dose levels of the main study were selected as follows: 1,250, 2,500, and 5,000 ug/mL for both short time (+/-S9) and continuous treatment (-S9). In addition, the positive and negative control groups were set. As a result of the main study, the frequency of cells with chromosome aberrations in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation was not statistically significantly different compared to the negative control

group (Table J.2). In the positive control group, the frequency of cells with structural chromosome aberrations in the short time treatments with and without metabolic activation and in the continuous treatment without metabolic activation was statistically significantly increased compared to the negative control group. Thus, it was concluded that 2’-FL was not clastogenic under the conditions of this study.

Table J.1. Number of Revertant Colonies (Main Study)

	Dose (µg/ plate)	Mean Number of Revertant Colonies[a]									
		TA98		TA100		TA1535		TA1537		WP2*uvrA* (pKM101)	
		1st*	2nd**	1st	2nd	1st	2nd	1st	2nd	1st	2nd
+ S9	0	35±1	35±1	122±2	117±3	13±1	12±1	22±1	20±1	114±1	117±3
	313	37±2	38±2	117±4	106±3	12±2	14±2	24±2	20±1	119±2	124±4
	625	35±1	39±2	112±6	104±4	11±1	13±1	25±3	19±2	118±4	125±4
	1,250	35±3	40±2	118±6	112±5	14±2	13±1	22±2	19±2	120±5	127±2
	2,500	36±3	37±1	126±2	111±3	11±2	10±1	20±3	23±1	117±4	125±4
	5,000	34±2	36±1	116±4	110±2	15±2	11±1	20±2	22±1	109±3	131±5
Positive Control + S9	Identity	2-AA		2-AA		2-AA		2-AA		2-AA	
	Dose (µg/ plate)	1.0		2.0		3.0		3.0		2.0	
	No. revertant colonies	368±15	347±3	961±16	942±10	176±5	174±6	225±2	238±4	397±5	517±19
- S9	0	25±1	22±1	102±5	96±5	15±1	16±1	9±1	9±1	98±2	104±3
	313	21±1	22±2	114±1	97±5	14±2	13±1	11±2	11±1	91±4	102±3
	625	20±2	23±1	111±5	94±5	16±0	17±2	11±2	8±1	103±4	94±2
	1,250	24±2	22±2	111±3	92±3	17±2	15±1	12±2	10±1	110±4	92±5
	2,500	25±1	24±1	122±7	105±2	15±3	14±1	10±1	9±2	102±3	113±2
	5,000	21±1	20±1	131±6	92±3	18±1	18±2	9±1	10±0	111±4	120±4
Positive Control - S9	Identity	2-NF		SA		SA		9-AA		4-NQO	
	Dose (µg/plate)	5.0		1.5		1.5		80.0		0.1	
	No. revertant colonies	718±5	739±10	738±8	736±9	580±4	585±11	566±7	607±8	423±1	581±20

[a]Mean number of revertant colonies presented as mean ± standard deviation (SD)

*1st main study results; **2nd main study results

Abbreviations: 2-AA= 2-aminoanthracene; 2-NF= 2-nitrofluorene; 9-AA= 9-aminoacridine; 4-NQO= 4-nitroquinoline N-oxide; SA= sodium azide

Table J.2. Results of *In Vitro* Mammalian Chromosomal Aberration Test (Main Study)

Test Substance	Dose (µg/mL)	S9 Mix	Trt-Rec Time (h)	Number of Cells with Structural Aberrations		Number of Cells with Numerical Aberrations
				Total (%)		Total (%)
				gap-	gap+	
Water	0	-	6-18	1 (0.3)	1 (0.3)	1 (0.3)
2’-FL	1,250	-	6-18	0 (0.0)	1 (0.3)	1 (0.3)
	2,500	-	6-18	0 (0.0)	0 (0.0)	1 (0.3)
	5,000	-	6-18	2 (0.7)	3 (1.0)	0 (0.0)
MMC	0.1	-	6-18	62 (20.7)*	64 (21.3)	0 (0.0)
Water	0	+	6-18	1 (0.3)	2 (0.7)	1 (0.3)
2’-FL	1,250	+	6-18	0 (0.0)	0 (0.0)	0 (0.0)
	2,500	+	6-18	0 (0.0)	0 (0.0)	0 (0.0)
	5,000	+	6-18	1 (0.3)	1 (0.3)	1 (0.3)
B[a]P	20	+	6-18	66 (22.0)*	68 (22.7)	1 (0.3)
Water	0	-	24-0	1 (0.3)	2 (0.7)	1 (0.3)
2’-FL	1,250	-	24-0	0 (0.0)	0 (0.0)	0 (0.0)
	2,500	-	24-0	0 (0.0)	0 (0.0)	1 (0.3)
	5,000	-	24-0	0 (0.0)	1 (0.3)	1 (0.3)
MMC	0.1	-	24-0	117 (39.0)*	119 (39.7)	0 (0.0)

Number of cells analyzed = 150 cells
Abbreviations: MMC = mitomycin C; B[a]P = benzo[a]pyrene; Trt-Rec time = treatment-recovery times; gap- = total number of cells with structural aberrations excluding gap; gap+ = total number of cells with structural aberrations including gap.
*Significant difference from a negative control by Fisher’s exact test, $p<0.01$.

In Vivo Mouse Micronucleus Test of APTech’s 2’-FL

This study was designed to evaluate the potential of the test substance, 2’-FL (purity, ≥94%), to induce micronuclei in bone marrow cells of CrlOri:CD1(ICR), SPF mice when the test substance was orally administered via gastric intubation twice at 24-hour intervals. In order to determine the high dose level of the main study, a dose range finding study was conducted. The high dose was set at 7,500 mg/kg, and it was sequentially diluted to produce 3 lower dose levels (5,000, 2,500, and 1,250 mg/kg). As a result, there were no clinical signs or mortality at any dose level of the test substance in male and female mice.

Therefore, the high dose level of the main study was set at 7,500 mg/kg and two additional lower dose levels (5,000 and 2,500 mg/kg) were produced. In addition, the positive and negative control groups were set. Because there was no mortality in either sex as a result of the dose range finding study, the main study was conducted with only

males, which are known to be susceptible to micronucleus induction. Twenty-five male mice aged 8 weeks were treated by oral gavage with 2’-FL dissolved in saline over 2 consecutive days before being sacrificed. Saline was used as a vehicle control. Mitomycin C (2 mg/kg, i.p.) was administered as the positive control. Clinical signs were recorded on Day 0 (immediately and at 2 hours after the 1st dosing), Day 1 (before the 2nd dosing, immediately and at 2 hours after the 2nd dosing), and Day 2. All doses were well tolerated, and no clinical signs were observed. Immediately following sacrifice, femurs were dissected from each animal and trimmed, and bone marrow cells were collected to evaluate the frequency of micronuclei.

No statistically significant increases in the incidence of micronucleated polychromatic erythrocytes (MNPCE) in polychromatic erythrocytes (PCE) were observed in any test substance groups compared with the negative control group (Table J.3). A significant increase in the incidence of MNPCE in PCE was observed in the positive control group compared with the negative control group. There were no statistically significant differences in the ratio of PCE to total erythrocytes in any test substance groups compared with the negative control value. Body weights of mice were comparable among the groups before and after treatment with the test substance. It was concluded that 2’-FL did not induce micronuclei in the bone marrow cells of mice under the conditions of this study.

Table J.3. Results of *In Vivo* Micronucleus Study (Main Study)

Groups		Dose (mg/kg)	Route	PCE/(PCE+NCE) (%)	MNPCE/PCE (%)
Negative Control	Water	0	P.O.	31.1±1.46	0.035±0.014
Test Substance	2’-FL	2,500	P.O.	33.0±1.79	0.030±0.033
		5,000	P.O.	32.5±1.47	0.040±0.014
		7,500	P.O.	31.7±1.24	0.060±0.014
Positive Control	MMC	2	I.P.	32.2±1.40	6.875±0.331

Mean±SD

I.P.= intraperitoneal; MMC= mitomycin C; MNPCE= micronucleated polychromatic erythrocyte; PCE= polychromatic erythrocyte; P.O.= per os; NCE= normochromatic erythrocyte

An Acute Oral Toxicity Study of APTech’s 2’-FL

An acute oral toxicity study was conducted with APTech’s 2′-FL (purity, ≥94%) in juvenile (7 days old) male and female Sprague-Dawley rats. The test groups consisted of three dose groups at dose levels of 2,500, 5,000, and 7,500 mg/kg bw and a control group (water), with 5 animals of each sex per group. All animals were monitored for clinical signs and body weight changes during the 14-day observation period after dosing. They were euthanized and subjected to gross necropsy at the end of the observation period.

One female was found dead at 7,500 mg/kg bw on day 2 after dosing. However, there were no test substance-related clinical signs and body weight changes in the other female pups in the 7,500 mg/kg bw dosing group. It was not considered to be test substance-related mortality since it was a natural death of the rat pup. In clinical signs, there were no abnormalities in the control and test groups. During the 14 day observation period, The body weight gain was significantly suppressed in the high dose male group, but not in females (control, low- vs. mid- vs high-dose: males - 53.1 vs. 50.5 vs. 51.5 vs. 45.0, $P<0.01$; females - 50.5 vs. 49.0 vs. 48.0 vs. 47.0, NS). At necropsy, there were no test substance-related gross findings in either sex at 2,500, 5,000, and 7,500 mg/kg. It was concluded that the mean lethal dose (LD_{50}) was greater than 7.5 g/kg bw, the highest dose tested.

Subchronic Oral Toxicity Study of APTech’s 2’-FL in Rats

Methods

The oral subchronic toxicity of 2’-FL (purity, ≥94%) was assessed in Sprague-Dawley [Crl:CD(SD)] rats and the reversibility of toxic effects was assessed following a 4-week recovery period. SD rats (n=10/sex/group) received 0, 2,500, 5,000, or 7,500 mg/kg/day 2’-FL via oral gavage for 90 days starting at day 7 of life. An additional 5 rats/sex/group received 0 or 7,500 mg/kg/day as the recovery group. At post-weaning on PND 21, all pups were separated individually in each cage. During the dosing and recovery periods, all animals were observed once daily for clinical signs and twice daily for mortality. Body weights and food consumption were also recorded. At weeks 12-13 for the main group and recovery weeks 3-4 for the recovery group, functional observations were performed for motor activity, grip strength, and auditory, visual, and proprioceptive stimuli. In all animals in the control and high-dose groups, ophthalmological examinations were conducted on both eyes at week 13. Urinalysis was conducted in 5 males and females per group from the main group and all animals from the recovery group. Fresh urine parameters included pH, protein, glucose, ketone body, bilirubin, occult blood, color and turbidity, and sediment. Urine volume and specific gravity were evaluated in the 24-hour urine sample.

Blood was collected from all surviving animals. For hematology, blood samples were placed in a vacutainer containing EDTA and evaluated for the following parameters: total erythrocyte count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), total leukocyte count (WBC), neutrophil (NEU), lymphocyte (LYM), monocyte (MONO), eosinophil (EOS), basophil (BASO), and reticulocyte (Reti). Blood samples mixed with 3.2% sodium citrate were centrifuged at 3,000 rpm for 10 minutes to obtain plasma to evaluate prothrombin time (PT) and activated partial thromboplastin time (APTT).

Blood samples from the abdominal aorta were centrifuged at 3,000 rpm for 10 minutes to obtain serum. Serum chemistry parameters included alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase

(ALP), gamma glutamyl transpeptidase (GGT), blood urea nitrogen (BUN), creatinine (Crea), total bilirubin (T-Bili), total bile acid (TBA), total protein (TP), albumin (Alb), A/G ratio, total cholesterol (T-Chol), triglycerides (TG), phosphorus (P), glucose (Glu), calcium (Ca), chloride (Cl), sodium (Na), and potassium (K).

All surviving animals in the main and recovery groups were sacrificed by exsanguination from the abdominal aorta on days 91 and 119, respectively. Complete gross postmortem examinations were completed. The following organ weights were recorded: brain, thymus, heart, liver, spleen, kidney, adrenal gland, testis, epididymis, ovary, uterus, and cervix. Histopathological examinations were conducted on the following organs and tissues: brain, pituitary gland, thyroid gland, parathyroid gland, thymus, lung (including bronchi), trachea, heart, liver, spleen, kidney, adrenal gland, salivary glands (submandibular, sublingual, and parotid), esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, testis, epididymis, prostate, seminal vesicle with coagulating gland, ovary, uterus (including cervix), vagina, urinary bladder, submandibular lymph node, mesenteric lymph node, eye, optic nerve, Harderian gland, mammary gland (inguinal), skin (inguinal), sternum (including bone marrow), femur (including bone marrow), skeletal muscle (thigh), sciatic nerve, spinal cord, and organs with gross lesions. Histopathological examinations were performed in the above-mentioned organs and tissues from control and high-dose groups, and dead animals. They were also performed on organs and tissues with macroscopic lesions from animals in the low- and mid-dose groups.

The study was conducted in accordance with the Good Laboratory Practice (GLP) regulations for nonclinical laboratory studies from the Korean Ministry of Food and Drug Safety and the Organization for Economic Co-operation and Development (OECD) guidelines (OECD 408).

Statistical Analysis

In the subchronic study, Bartlett's test was used to determine the homogeneity of variance (significance level of 0.05). ANOVA was performed on homogeneous data, and, if significant, Dunnett's test was applied for multiple comparisons (significance levels at 0.05 and 0.01, two-tailed). For the dosing period of the subchronic study, the Kruskal-Wallis test was used on heterogeneous data, and, if significant, the Steel test was applied for multiple comparisons (significance levels at 0.05 and 0.01, two-tailed). For the recovery group, the Folded F-test was performed for homogeneity of variance (significance level at 0.05). The Student t-test was conducted if the variances of two populations were homogeneous, and the Aspin-Welch t-test was performed if the variances were heterogeneous (significance levels at 0.05 and 0.01, two-tailed).

Results

1. Body weight

During the dosing period, there were statistically significant decreases in body weight in males in the 5,000 mg/kg bw/day group on day 11 (47.5±2.8 vs. 44.7±2.6 g,

p<0.05 by Dunnett's t-test) and the 7,500 mg/kg/day group on day 4 (27.6±1.7 vs. 26.0±0.8 g, p<0.05 by Steel test). These changes were not considered test substance-related since there was no dose dependency and they were temporary, with little difference compared to the control group. Starting day 15 after dosing and during the recovery period, no significant differences in body weights were observed in either sex among the groups (control vs. low- vs. mid- vs. high dose: males at day 90, 602.2 vs. 625.1 vs. 581.5 vs. 647.2 g, NS; females at day 90, 337.7 vs. 323.4 vs. 325.9 vs. 321.5 g, NS; Figures J.1 and J.2). During the recovery period, there was no test substance-related effect in both sexes in the 7,500 mg/kg/day group.

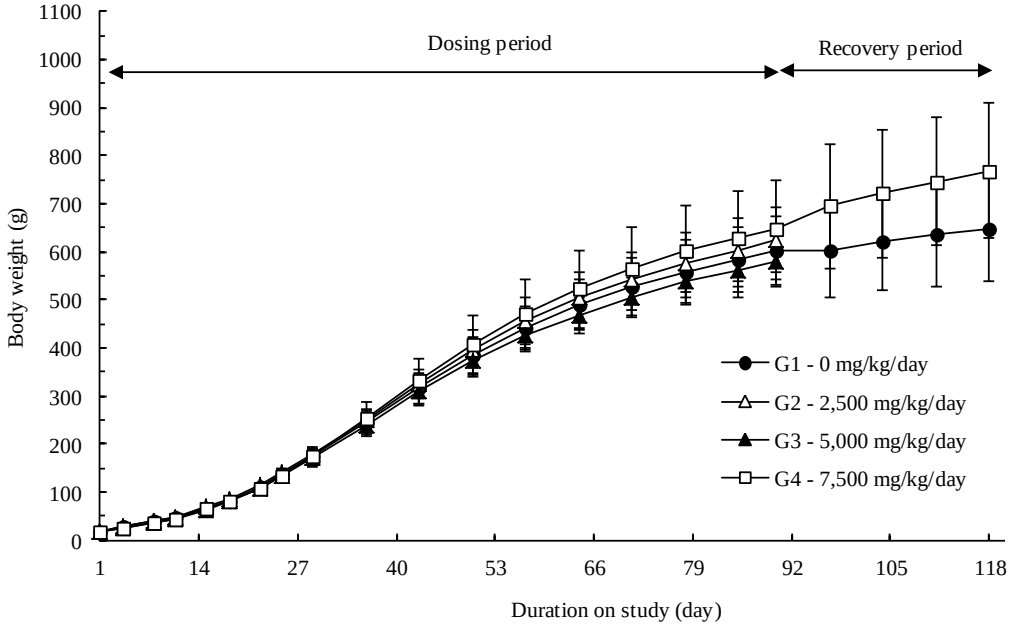


Figure J.1. Body Weights in Male SD Rats

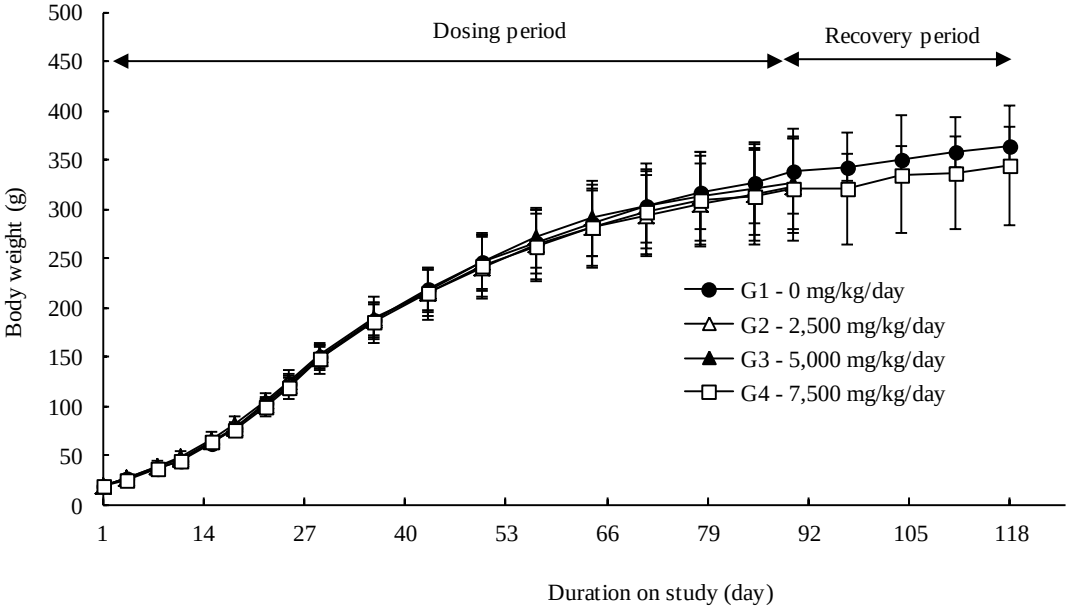


Figure J.2. Body Weights in Female SD Rats

2. Food consumption

The dosing period resulted in no effect on food consumption in both sexes in the test groups compared to the control group. At 13 weeks, the food intake ranged from 36.1 to 39.9 g/day (NS) in male rats and 24.5 to 27.8 g/day (NS) in female rats with no dose responses noted (data not shown).

3. Functional observation and ophthalmological examinations

Functional observations of the male and female rats in the test groups in the main group and the 7,500 mg/kg/day group in the recovery group indicated no effects in visual response, proprioceptive stimuli, auditory response, pain response, aerial righting reflex, hindlimb landing foot splay, grip strength, and motor activity. There were statistically significant decreases in hindlimb grip strength in the female test groups of the main group, but these were not considered of toxicological concern since the differences were small (G1 vs. G2 vs. G3 vs. G4: 0.484±0.068 vs. 0.408±0.060 vs. 0.412±0.058 vs. 0.405±0.062 kgf, $p<0.05$). There were no ocular abnormalities found in the ophthalmological examinations in males and females of the control and 7,500 mg/kg/day groups.

4. Urinalysis

Urinalysis of glucose, ketone body, bilirubin, pH, protein, and occult blood demonstrated no treatment-related effects in males and females among the groups at the end of the 13-week treatment and 4-week recovery.

5. Hematology

No clinically significant treatment-related effects were observed in the hematological parameters among the groups at the end of 13-week treatment (Table J.4). A significant increase in MONO was observed in the high-dose male group (7.6±1.6 vs. 10.6±2.3%, $p<0.05$) but not in any of the female groups. However, the male group values were within the normal range and were not clinically significant. There was a significant decrease in EOS (1.3±0.4 vs. 0.9±0.2%, $p<0.05$) in females in the 5,000 mg/kg/day group. However, the values were within the normal range and no dose responses were observed; thus, the changes were not considered of toxicological concern. Significant decreases in PT were observed in females in the 5,000 mg/kg/day (18.5±0.7 vs. 17.7±0.7 sec, $p<0.05$) and 7,500 mg/kg/day groups (18.5±0.7 vs. 17.5±0.7 sec, $p<0.05$). Although statistically significant, the changes were not considered clinically significant since the differences were of a small magnitude and the values were within the range of the historical reference data. In addition, no test substance-related effects were observed in clinical parameters at the end of the 4-week recovery in the high-dose group (data not shown).

6. Clinical chemistry

No clinically significant treatment effects were observed in the clinical parameters of the male and female groups at the end of the 13-week treatment (Table J.5). There were significant decreases in alanine aminotransferase (ALT) in the mid-dose female group (23.1±6.0 vs. 16.4±2.6 U/L, $p<0.05$) and gamma glutamyl transpeptidase (GGT) in the high-dose female group (0.53±0.24 vs. 0.24±0.11 U/L, $p<0.05$). In males, no statistically significant

differences were noted in these enzyme values. A significant increase in total cholesterol (T-Chol) was observed in the female test groups but not the male test groups compared to the control group (control vs. low-dose vs. mid-dose vs. high-dose: 70±14 vs. 88±16 vs. 94±21 vs. 98±14 mg/dL, $p<0.01$). In the male rats, statistically significant changes in creatine, P, and Cl were noted in the mid- and high-dose groups, but not in any of female groups. However, all the values listed in Table 6 were within the historical normal ranges or the differences were small in magnitude; thus, they were not considered as toxicological concern. In addition, no test substance-related effects were observed in the clinical parameters at the end of the 4-week recovery in the high-dose group (data not shown).

7. Organ weights

The administration of 2’-FL had no effects on the absolute and relative organ weights of males and females in the control and test groups at the end of the 13-week administration of 2’-FL (data not shown). No test substance-related effects were observed in the clinical parameters at the end of 4-week recovery in the high-dose group (data not shown).

Table J.4. Mean Hematological Parameters in the Main Group

Parameters	Male				Female			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)
RBC ($x10^6$/µL)	8.50±0.33	8.47±0.41	8.54±0.30	8.48±0.55	8.11±0.20	7.88±0.23	8.00±0.35	7.91±0.24
HGB (g/dL)	15.8±0.4	15.8±0.7	15.7±0.5	15.7±0.7	15.5±0.3	15.4±0.3	15.4±0.5	15.2±0.3
HCT (%)	44.2±1.2	44.2±1.7	44.0±1.5	43.9±2.1	42.8±1.2	42.4±1.0	42.8±1.5	42.3±1.0
RBC Indices								
MCV (fL)	52.0±0.9	52.2±1.6	51.6±0.4	51.8±1.2	52.8±0.6	53.8±1.1	53.6±1.7	53.6±1.3
MCH (pg)	18.6±0.4	18.6±0.7	18.4±0.2	18.5±0.5	19.1±0.3	19.5±0.4	19.3±0.6	19.3±0.4
MCHC (g/dL)	35.8±0.3	35.7±0.3	35.7±0.4	35.7±0.4	36.1±0.4	36.3±0.3	36.0±0.2	36.0±0.6
PLT ($x10^3$/µL)	982±97	978±105	974±90	960±67	930±106	869±80	872±99	947±103
Reti (%)	3.81±0.29	3.77±0.43	3.45±0.45	3.39±0.39	3.32±0.53	3.38±0.34	3.46±0.52	3.37±0.52
WBC ($x10^3$/µL)	11.21±3.00	10.86±1.77	11.36±3.20	10.74±2.44	7.24±3.50	5.67±2.00	5.18±1.63	5.28±1.81
WBC Differential Counting (%)								
NEU	21.6±6.5	18.1±3.9	18.2±5.4	17.6±4.5	15.6±5.2	15.8±4.6	15.8±7.9	19.6±8.1
LYM	69.2±6.4	70.7±4.5	72.3±6.5	70.2±4.6	75.4±6.3	75.9±6.3	76.4±7.4	72.6±7.9
MONO	7.6±1.6	9.7±2.8	8.4±2.4	10.6±2.3*	7.4±2.3	6.9±2.0	6.7±2.4	6.5±0.9
EOS	1.2±0.4	1.1±0.3	0.9±0.2	1.2±0.5	1.3±0.4	1.2±0.4	0.9±0.2*	1.1±0.4
BASO	0.3±0.2	0.4±0.2	0.2±0.1	0.3±0.1	0.3±0.2	0.2±0.1	0.2±0.1	0.2±0.1
PT (sec)	18.5±0.8	17.5±1.1	17.6±0.9	17.9±0.9	18.5±0.7	18.0±0.8	17.7±0.7*	17.5±0.7*
APTT (sec)	15.2±1.7	14.7±2.2	15.3±0.9	15.3±1.3	14.6±1.1	14.4±1.1	15.0±1.0	14.8±0.9

Mean±SD

*Significantly different from control by Dunnett's t-test, $p<0.05$.

Abbreviations: RBC= total erythrocyte count; HGB= hemoglobin; HCT= hematocrit; MCV= mean corpuscular volume; MCH= mean corpuscular hemoglobin; MCHC= MCH concentration; PLT= platelet count; Reti= reticulocyte; WBC= total leukocyte count; NEU= neutrophil; LYM= lymphocyte; MONO= monocyte; EOS= eosinophil; BASO= basophil; PT= prothrombin time; APTT= activated partial thromboplastin time.

Table J.5. Clinical Chemistry in Main Group

Parameters	Male				Female			
	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)	G1 (0)	G2 (2,500)	G3 (5,000)	G4 (7,500)
ALT (U/L)	24.6±6.7	23.5±4.9	22.9±5.3	32.4±25.6	23.1±6.0	18.4±4.7	16.4±2.6*	19.7±6.0
AST (U/L)	72.1±15.7	68.4±10.6	69.7±13.5	85.5±36.2	62.5±11.2	63.0±7.9	55.4±6.1	59.2±11.4
ALP (U/L)	305.6±63.8	329.9±36.7	306.2±61.2	314.4±68.9	214.1±67.4	197.6±40.6	213.8±83.1	236.7±123.3
GGT (U/L)	0.27±0.17	0.18±0.11	0.24±0.13	0.22±0.08	0.53±0.24	0.51±0.20	0.42±0.30	0.24±0.11*
Glu (mg/dL)	160±25	159±15	152±12	151±16	148±13	147±17	157±17	163±18
BUN (mg/dL)	12.8±1.7	12.1±0.9	11.8±1.6	10.9±1.8	13.0±1.8	14.3±1.9	12.9±1.6	13.8±2.2
Crea (mg/dL)	0.45±0.06	0.45±0.04	0.41±0.03	0.40±0.04*	0.47±0.03	0.51±0.04	0.46±0.04	0.47±0.02
T-Bili (mg/dL)	0.08±0.03	0.06±0.02	0.06±0.01	0.06±0.02	0.07±0.02	0.07±0.02	0.05±0.01	0.06±0.02
T-Chol (mg/dL)	90±13	97±26	75±14	74±21	70±14	88±16*	94±21**	98±14**
TG (mg/dL)	32±15	69±35#	58±59	66±56	17±7	18±10	20±7	40±43
TP (g/dL)	5.9±0.2	6.1±0.3	5.9±0.2	5.9±0.2	6.0±0.3	6.0±0.3	6.0±0.4	6.1±0.3
Alb (g/dL)	2.3±0.1	2.4±0.1	2.3±0.1	2.3±0.1	2.6±0.2	2.6±0.2	2.6±0.2	2.7±0.1
A/G ratio	0.64±0.06	0.64±0.04	0.63±0.05	0.64±0.05	0.74±0.02	0.76±0.04	0.79±0.08	0.82±0.07
P (mg/dL)	6.25±0.26	6.61±0.50	6.79±0.38*	6.82±0.63*	5.18±0.67	5.57±0.41	5.33±0.40	4.95±0.45
Ca (mg/dL)	10.0±0.4	10.4±0.3	10.1±0.4	10.1±0.6	9.6±0.5	9.9±0.4	10.0±0.5	9.8±0.5
Na (mmol/L)	136.3±2.1	135.8±1.3	135.5±1.9	135.5±3.7	136.5±1.1	136.7±1.0	136.1±2.2	135.2±1.5
K (mmol/L)	3.69±0.33	3.79±0.20	3.80±0.19	3.97±0.16	3.66±0.29	3.52±0.30	3.75±0.32	3.65±0.23
Cl (mmol/L)	106.2±0.8	105.8±1.0	105.8±1.9	104.5±1.1*	108.8±1.1	108.2±0.9	107.8±1.7	107.1±1.5

Mean±SD

*Significantly different from control by Dunnett's t-test, $p<0.05$; **$p<0.01$.

#Significantly different from control by Steel test, $p<0.05$.

Abbreviations: ALT= alanine aminotransferase; AST= aspartate aminotransferase; ALP= alkaline phosphate; GGT= gamma glutamyl transpeptidase; Glu= glucose; BUN= blood urea nitrogen; Crea= creatinine; T-Bili= total bilirubin; T-Chol= total cholesterol; TG= triglyceride; TP= total protein; Alb= albumin; P= phosphorus; Ca= calcium; Na= sodium; K= potassium; Cl= chloride.

8. Necropsy and histopathology

There were no treatment-related gross visible findings or lesions in the males or females during the dosing and recovery periods (data not shown). No histopathological effects associated with the administration of the test substance were seen in males and females in the high-dose group. In the 5,000 mg/kg/day group, one male was found dead on Day 72. It was considered a sudden death not associated with morphological changes common in SD rats. It might have been associated with technical gavage error, but not with the test substance treatment.

Soft stool or diarrhea were often observed in both sexes in the 7,500 mg/kg/day group on day 26 until the end of dosing, but not during the recovery period, except on day 91, the day before the final dosing. However, there were no test substance-related changes in body weight, food consumption, gross findings at necropsy, and histopathological results. Therefore, it was considered to have little toxicological significance. A transient diarrhea (or relieving of constipation symptoms) is often associated with high intake of non-digestible carbohydrate or dietary fiber ingredients (Institute of medicine [IOM], 2002) and is not considered as toxicological concern, especially because no test substance-related changes were found in body weight, food consumption, or gross findings at necropsy and histopathology. Despite gastrointestinal discomfort including diarrhea associated with high intake of dietary fiber, the IOM has not established Tolerable Upper Intake Levels (UL) for dietary fiber.

In addition, macroscopic and microscopic examinations found no treatment-related adverse effects in the high-dose group at the end of the 13-week treatment. The incidence of histopathological changes was comparable among control and high dose groups. Overall, no treatment-related abnormalities were observed from macroscopic and histopathological examinations of other organs.

Conclusion

Based on the observations and analyses in these studies, it was concluded that 2'-FL produced by fermentation via genetically engineered *Corynebacterium glutamicum* APC199, a non-toxigenic and non-pathogenic strain, did not produce any significant changes in physical, physiological, biochemical, hematological, or histopathological parameters in any of the doses used in the 90-day toxicity studies. The NOAEL value for the 13-week oral toxicity study in male and female rats was 7,500 mg/kg, the highest dose tested. The 2'-FL that was the subject of this study shows toxicity profiles that are comparable to other 2'-FLs prepared by different manufacturing methods and other human milk oligosaccharides.

Reference

Institute of Medicine. Dietary Reference Intakes for energy, carbohydrates, fiber, fat, fatty acids, cholesterol, protein, and amino acids. National Academy Press, Washington, DC. 2002.

Appendix K. *In vitro* Safety Tests for *Corynebacterium glutamicum* APC199

Objective

To determine the safety of the production microorganism via simple *in vitro* tests, such as biogenic amine production and hemolytic and gelatinase activities.

In vitro Biogenic Amine Test

C. glutamicum, grown at 30°C for 24 hours in BHI broth, was streaked out onto special medium with lysine, tyrosine, histamine, and ornithine as precursor amino acids using the method described in Bover-Cid and Holzapfel (1999) and incubated for 48 hours at 30°C to detect biogenic amine production (cadaverine, tyramine, histamine and putrescine, respectively). *E. coli* ATCC 25922 was used as a positive control.

C. glutamicum as shown to be negative for four different biogenic amine productions (histamine, cadaverine, tyramine, and putrescine) at 30°C (Table K.1).

Table K.1. Biogenic amine production activity of *C. glutamicum* APC 199 and *E. coli* ATCC 25922.

Strain	Histamine	Cadaverine	Tyramine	Putrescine
Corynebacterium glutamicum APC 199	Negative	Negative	Negative	Negative
Escherichia coli ATCC 25922 (positive control)	Positive	Positive	Positive	Positive

In vitro Hemolysis Test

C. glutamicum APC199 was grown at 30°C for 24 hours in BHI broth and then streaked onto 5% sheep blood agar (Hanil Komed) and incubated for 24 hours at 30 °C. Alpha (α) hemolysis was considered as the partial decomposition of the hemoglobin of the red blood cells (but does not represent true hemolysis) and beta (β) hemolysis as the complete breakdown of the hemoglobin of the red blood cells observed as a clear zone in the agar plate, while gamma (γ) hemolysis was considered as the lack of hemolysis. *Staphylococcus aureus* ATCC 6538 was used as a positive control. *C. glutamicum* showed a negative reaction for hemolysis (Table K.2).

Table K.2. Hemolysis activity of *C. glutamicum* and *S. aureus* ATCC 6538.

Strain	Hemolysis activity
Corynebacterium glutamicum APC199	Gamma
Staphylococcus aureus ATCC 6538 (positive control)	Beta

In vitro Gelatinase Activity test

The basic protocol was followed according to ASM Science Recommendation (Dela Cruz and Torres, 2012). *C. glutamicum* strain, grown at 30°C for 24 hours in BHI broth, was inoculated in a gelatin medium with a loop and incubated at 30°C for up to 1 week, and checked daily for gelatin liquefaction. Gelatin normally liquefies at 28°C and above, so to confirm that liquefaction was due to gelatinase activity, the tubes are placed in a refrigerator for 15 to 30 minutes. Afterwards, tubes are tilted to observe if gelatin has been hydrolyzed. Hydrolyzed gelatin will result in a liquified medium even after exposure to cold temperature. *Bacillus cereus* ATCC 11778 was used as a positive control. *C. glutamicum* APC199 showed a negative reaction for the gelatinase test (Table K.3).

Table K.3. Gelatinase test for *C. glutamicum* APC199 and *B. cereus* ATCC 11778

Strain	Gelatinase test
Corynebacterium glutamicum strain	Negative
Bacillus cereus ATCC 11778 (positive control)	Positive

Detection of virulence genes in *C. glutamicum* test strain

The whole genome sequence of *C. glutamicum* APC 199 was analyzed to detect known major virulence genes of the pathogenic *Bacillus cereus*. Virulence genes include aggregation substance, cytolysin, cytotoxin K, enterococcal surface protein, endocarditis antigen, adhesion of collagen, enterotoxin, gelatinase, hemolysin, hyaluronidase, and cereulide. As the whole genome sequencing results of *C. glutamicum* with *B. cereus* ATCC14579 were compared, no toxigenic genes were found in *C. glutamicum* APC 199 while various toxigenic genes were detected in *B. cereus* ATCC14579 that implies the safety of *C. glutamicum* APC199 as well as the absence of potentially toxigenic genes (Table K.4).

Table K.4. List of major virulence genes in *B. cereus* compared to *C. glutamicum* strain

Potential virulence genes	*C. glutamicum* strain	*B. cereus* ATCC14579
Aggregation substance (*asa1*)	Negative	Negative
Cytolysin (*CylA*)	Negative	Positive
Cytotoxin K (*cytK*)	Negative	Positive
Enterococcal surface protein (*esp*)	Negative	Negative
Endocarditis antigen (*efa*A)	Negative	Negative
Adhesion of collagen (*ace*)	Negative	Positive
Enterotoxin	Negative	Positive
Non-hemolytic enterotoxin (*nhe*)	Negative	Positive
Gelatinase (*coccolysin, gelE*)	Negative	Negative
Hemolysin (*hbl*)	Negative	Positive
Hyaluronidase (*hyl*)	Negative	Negative
Cereulide (*ces*)	Negative	Negative

Conclusions

C. glutamicum APC199 did not produce biogenic amines and had no hemolytic and gelatinase activities. The whole genome sequencing analysis also revealed that *C. glutamicum* APC199 was negative for major virulence genes. Thus, it is concluded that *C. glutamicum* APC 199 is non-toxigenic and non-pathogenic.

Appendix L. Allergenic Potential of Introduced Gene Products

Typically, sequence homology searches comparing the predicted structure of introduced proteins to known allergens in a database are conducted using various algorithms, such as FASTA. As recommended by FAO/WHO (2001), IgE cross-reactivity between a novel protein and a known allergen is considered a possibility when there is more than 35% identity over a segment of 80 or greater amino acids. For introduced proteins, the allergenic potential was screened using the database, http://allergenonline.org/databasefasta.shtml (March 23, 2018 version). Allergen online was used to compare FASTA sequences of each introduced protein to the protein sequences in the databases. Allergen online searches were conducted using default settings, and searches were conducted for matches to 80 amino acid sequence segments (sliding window) and 8-mer sequence alignments. In accordance with Codex guidelines, FASTA also was used to search for 80 amino acid sliding window segments aligning with a match ≥35% identity to a protein in the allergen database (Codex Alimentarius Commission, 2003). In addition, eight contiguous amino acid matches between a novel protein and a known allergen(s) are routinely used to identify sequences that may represent linear epitopes.

None of introduced gene products [GDP-L-fucose synthase (WcaG), GDP-D-mannose 4,6-dehydratase (Gmd), lactose permease (LacY), and fucosyltransferase (α-1,2-ft)] have homology in amino acid sequences with those of allergenic proteins.

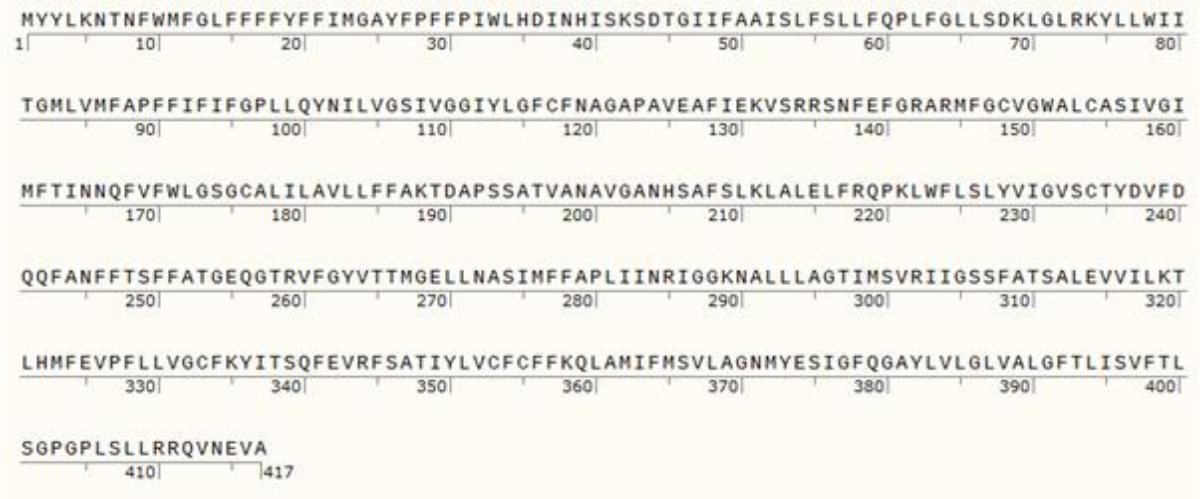
An introduced gene product, GDP-L-fucose synthase (WcaG), consists of 321 amino acids, and the amino acid sequences is as follows:

```
1      MSKQRVFIAG HRGMVGSAIR RQLEQRGDVE LVLRTRDELN LLDSRAVHDF FASERIDQVY
61     LAAAKVGGIV ANNTYPADFI YQNMMIESNI IHAAHQNDVN KLLFLGSSCI YPKLAKQPMA
121    ESELLQGTLE PTNEPYAIAK IAGIKLCESY NRQYGRDYRS VMPTNLYGPH DNFHPSNSHV
181    IPALLRRFHE ATAQNAPDVV VWGSGTPMRE FLHVDDMAAA SIHVMELAHE VWLENTQPML
241    SHINVGTGVD CTIRELAQTI AKVVGYKGRV VFDASKPDGT PRKLLDVTRL HQLGWYHEIS
301    LEAGLASTYQ WFLENQDRFR G
```

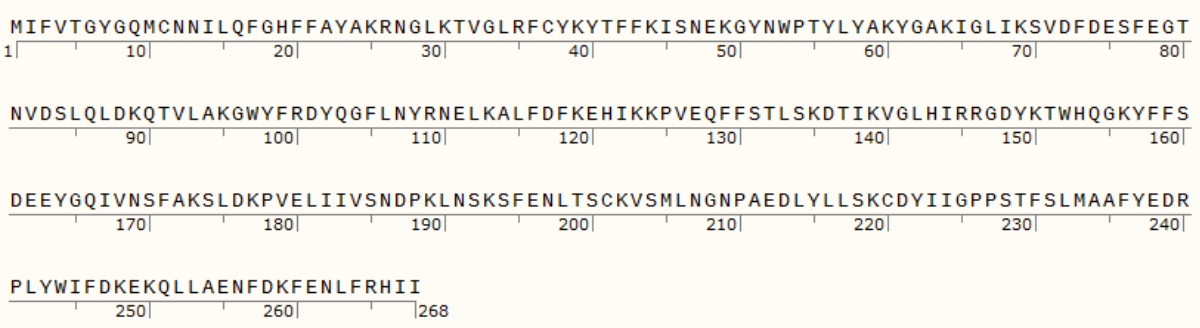
An introduced gene product, GDP-D-mannose 4,6-dehydratase (Gmd), consists of 373 amino acids, and the amino acid sequences is as follows:

```
1      MSKVALITGV TGQDGSYLAE FLLEKGYEVH GIKRRASSFN TERVDHIYQD PHTCNPKFHL
61     HYGDLSDTSN LTRILREVQP DEVYNLGAMS HVAVSFESPE YTADVDAMGT LRLLEAIRFL
121    GLEKKTRFYQ ASTSELYGLV QEIPQKETTP FYPRSPYAVA KLYAYWITVN YRESYGMYAC
181    NGILFNHESP RRGETFVTRK ITRAIANIAQ GLESCLYLGN MDSLRDWGHA KDYVKMQWMM
241    LQQEQPEDFV IATGVQYSVR QFVEMAAAQL GIKLRFEGTG VEEKGIVVSV TGHDAPGVKP
301    GDVIIAVDPR YFRPAEVETL LGDPTKAHEK LGWKPEITLR EMVSEMVAND LEAAKKHSLL
361    KSHGYDVAIA LES
```

An introduced gene product, lactose permease (LacY), consists of 417 amino acids, and the amino acid sequences is as follows:



An introduced gene product, α-1,2-fucosyltransferase (α-1,2-FT), consists of 268 amino acids, and the amino acid sequences is as follows:



Appendix M. Summary of Opinions of the Expert Panel

An independent panel of experts (Expert Panel), qualified by scientific training and experience to evaluate the safety of food and food ingredients, was requested by Advanced Protein Technologies (APTech) to evaluate the safety and Generally Recognized as Safe (GRAS) status of the use of 2'-fucosyllactose (2'-FL) in infant formula and other foods. APTech intends to provide 2'-FL to manufacturers of non-exempt (term) infant formulas and other food manufacturers for use as a food ingredient in term infant formulas and in other foods. The intended use levels are the same as for another approved use (GRN 735) for incorporation of 2'-FL in infant formulas and other foods. 2'-FL is currently marketed for use in infant formulas and foods for human consumption.

A detailed review based on the existing scientific literature and other pertinent information on the safety of 2'-FL was conducted by NutraSource, Inc. (NutraSource) and is summarized in the attached safety assessment dossier. After the Expert Panel members reviewed the dossier prepared by NutraSource and other pertinent information, they participated in a pre-submission meeting with FDA via a WebEx conference call on February 10, 2020. Subsequently, the Expert Panel members convened on February 14, 2020 via teleconference. Based on an independent, critical evaluation of all of the available information and discussions during the February 14, 2020 teleconference, the Expert Panel unanimously concluded that the intended uses described herein for APTech's 2'-FL, meeting appropriate food-grade specifications as described in the supporting dossier (Determination of The Generally Recognized As Safe [GRAS] Status of 2'-Fucosyllactose as a Food Ingredient) and manufactured according to current Good Manufacturing Practices (cGMP), are GRAS based on scientific procedures.

A summary of the basis for the Expert Panel's conclusion is provided below.

<u>Description</u>

The common names of the subject of this GRAS assessment are 2'-fucosyllactose or 2'-*O*-fucosyllactose and are often abbreviated as 2'-FL, 2-FL, or 2FL. 2FL is a trisaccharide consisting of L-fucose and lactose (D-galactose and D-glucose) (chemical formula: $C_{18}H_{32}O_{15}$; molecular mass: 488.44 Da; CAS No 41263-94-9). The monosaccharide L-fucose is linked to the disaccharide D-lactose by an α-(1→2) bond. The mean concentrations of 2'-FL in human milk are clustered around 2.4 g/L (Castanys-Muñoz et al., 2013).

Manufacturing Process

2'-FL is produced through the enzymatic transfer of fucose to lactose in an α-1,2-linkage. The reaction is catalyzed by fucosyltransferase present in a genetically modified *Corynebacterium glutamicum* APC199 strain. The main production process of APTech's 2'-FL consists of two steps. The first step is fermentation for the production of 2'-FL using genetically engineered *Corynebacterium glutamicum* APC199. The major components of the fermentation medium are yeast extract, glucose, and mineral sources. During the fermentation with a non-pathogenic and non-toxigenic *Corynebacterium glutamicum* APC199 strain, 2'-FL is biosynthesized inside the cells and exported into the culture broth.

Upon completion of fermentation, the culture supernatant containing 2'-FL after microfiltration is de-colorized by treatment with activated carbon to remove colorants present in the supernatant. Subsequently, macromolecules are removed by ultrafiltration with a 1.5 kDa molecular weight cut-off (MWCO) membrane. The resulting ultrafiltration permeate is purified through a nanofiltration process. This nanofiltration can remove very small molecules (MWCO 300 Da), such as monosaccharides, disaccharides, amino acids, organic acids, minerals, and salts. Next, an additional activated carbon treatment is performed to remove traces of coloring and organic materials, followed by an ion-exchange chromatography step to remove charged compounds (e.g., peptides, organic acids, and inorganic salts). Potential microbial contaminants during the purification processes are then removed by microfiltration with a 0.25 μm membrane, and the solution is concentrated prior to the crystallization process. Crystallization is induced by adding an anti-solvent (acetic acid or ethanol). Formed 2'-FL crystals are washed with the fresh anti-solvent and dried under vacuum to obtain a high-purity white powder form of 2'-FL.

All raw materials used in the manufacturing process are suitable food-grade, and are used in accordance with applicable US Federal Regulations. All processing aids are suitable for use in food manufacturing and are compliant with applicable US Federal Regulations. APTech's fermentation process does not use antibiotics or inhibitors and the manufacturing process does not use strong organic solvents or other toxic substances.

The production strain used in the fermentation is a genetically modified *Corynebacterium glutamicum* APC199. The parental strain *Corynebacterium glutamicum* ATCC13032 (NCIMB 10025) was isolated from sewage as a producer of glutamate as early as the 1950's and was deposited in the American Type Culture Collection (ATCC) as ATCC13032. *Corynebacterium glutamicum* APC199 was modified from *Corynebacterium glutamicum* ATCC13032 by inserting a pFP110 plasmid and is deposited under the Korean Collection for Type Cultures (KCTC) as KCTC 13735BP.

Identification, Specifications, and Analytical Values

Analytical data on multiple batches confirmed the purity of the product (>94%) and its manufacturing consistency by meeting specifications as demonstrated in the certificates of analysis. APTech's 2'-FL is substantially equivalent to the 2'-FL ingredients produced by other manufacturers that received FDA's no question letters. APTech's 2'-FL is chemically identical to reference standards as confirmed by high performance anion exchange chromatography (HPAEC), mass spectroscopy, and ^{1}H-NMR and ^{13}C-NMR spectroscopy. The product is ≥94% pure on a dry weight basis, as measured by high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The associated carbohydrate by-products (0.1% lactose, 0.34% difucosyllactose, 1.05% glucose, and 0.69% galactose) are similar to the other products and all are common components of breast milk and/or infant formula.

APTech's 2'-FL is chemically and structurally identical to the 2'-FL found in human milk. In addition, APTech's 2'-FL is chemically and structurally identical to synthetic 2'-FL preparation, for which FDA issued a 'no question' letter.

Stability

APTech has completed a 39-week accelerated storage and 39-week shelf stability study on its 2'-FL produced via genetically engineered *Corynebacterium glutamicum* APC199. The 2'-FL samples used in this stability study were in 2 forms: 1) powder form, and 2) 45% in aqueous solution. At accelerated conditions (40°C at a relative humidity of 75%), 100.5% recovery was reported at 39 weeks when compared to the baseline value. The data indicated that 2'-FL produced via genetically engineered *Corynebacterium glutamicum* APC199 is as stable as other 2'-FL ingredients manufactured by other processes.

Intended Use and Exposure Estimates

APTech intends to use 2'-FL as an ingredient in the following food categories: whey-, milk-, and/or soy-based, non-exempt infant formulas for term infants at a maximum level of 2.4 g/L of formula as consumed (ready-to-drink or reconstituted formula prepared from powder); formulas for toddlers and children aged 12 to 36 months at a maximum level of 2.4 g/L of formula as consumed (ready-to-drink or reconstituted formula prepared from powder); foods for infants and toddlers at levels of 0.24 -1.2 g/serving; and the following food categories at levels of 0.28 - 1.2 g/serving: 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 and milk products, processed fruits and fruit juices, and sweet sauces, toppings, and syrups.

From the use of 2'-FL in only infant formulas (2.4 g/L of ready-to-drink and/or reconstituted formula prepared from powder), in all-user infants aged 0 to 11.9 months old, the estimated mean and 90th percentile intakes of 2'-FL were determined to be at 1.87 and 2.78 g/person/day, respectively. On a body weight basis, these intakes were determined to be 258.7 and 431.3 mg/kg bw/day, respectively. The all-user estimated mean and 90th percentile intakes of 2'-FL were greatest in infants aged 3 to 5.9 months old at 2.04 and 2.93 g/person/day, respectively. On a body weight basis, the greatest intake was observed to occur in infants aged 0 - 2.9 months at 347.8 and 541.9 mg/kg bw/day, respectively.

Under the intended use of 2'-FL from the use of infant formula and other foods, the mean and 90th percentile EDIs of 2'-FL in all-users of all ages were determined to be 1.70 and 3.54 g/person/day, respectively. On a body weight basis, the mean and 90th percentile EDIs were determined to be 36 and 80 mg/kg bw/day, respectively, in all-users of all ages. The highest intake was observed to occur in male teenagers with the highest 90th percentile intake at 4.29 g/person/day. Of all-users, infants aged 0 to 5.9 months were estimated to have the highest mean and 90th percentile EDIs of 315 and 532 mg/kg bw/day, respectively. These EDIs are within safe intake levels.

Human infants have been exposed to 2'-FL via human breast milk. The EDIs of the 2'-FL for the proposed uses at their respective maximum use levels are unlikely to exceed the high intake level of 2'-FL in breastfed infants per kg bw because the maximum use level of 2'-FL in infant formula is similar to the mean concentration of 2'-FL in human milk (approximately 2.4 g/L). In addition, the proposed use levels are similar to those described in another 2'-FL GRAS notice. Thus, the intake of 2'-FL for the proposed uses at their respective maximum use levels can be considered safe. The EDI assessments are based on the assumption that APTech's 2'-FL will replace currently marketed 2'-FL. Thus, cumulative exposures are not expected to change.

Safety Evaluation

Metabolism

It is generally accepted that most of the HMOs, including 2'-FL, resist the pH of the stomach and are resistant to enzymatic hydrolysis in the small intestine to reach the large intestine intact. Thus, the majority of 2'-FL will pass through the intestinal tract and enter the colon intact, and will be transported intact to the large intestine and subjected to partial fermentation by the indigenous microbiota populations within the gastrointestinal tract (Brand-Miller et al., 1998).

Pre-clinical Studies

2’-FL has been evaluated by *in vitro* and *in vivo* genotoxicity studies, acute and subacute oral toxicity studies in rats and pigs, and subchronic toxicity studies in rats. 2’-FL was reported as non-mutagenic and non-clastogenic in all studies conducted.

Two published subchronic studies of 2’-FL were identified from the literature. In a subchronic gavage study by Coulet et al. (2014), the No Observed Adverse Effect Level (NOAEL) was determined to be 5,000 mg/kg bw/day for synthetic 2’-FL (manufacturer-Glycom) in male and female Wistar [Crl:Wl(Han)] rats. In this study, 2’-FL was administered via gavage in a juvenile adapted sub-chronic rat study at dose levels of 0, 2,000, 5,000, and 6,000 mg/kg bw/day for 13 weeks, along with fructooligosaccharide (FOS) as a reference high-dose control at 6,000 mg/kg bw/day. No treatment-related adverse effects were noted. The exception was that one male and one female rat in the 6,000 mg/kg bw/day 2'-FL dose group, and two males and one female in the 6,000 mg/kg bw/day FOS dose group died during the treatment period. One female in the 6,000 mg/kg bw/day FOS group died during the recovery period. The authors stated that the deaths in the highest dose group could not be definitely determined to be not treatment-related although there was no evidence of a direct toxic effect. Oral administration up to 5,000 mg/kg bw/day to rats over 90 days was not associated with any adverse effects on any parameters tested. Thus, the authors concluded that the NOAEL for 2'-FL was 5,000 mg/kg bw/day in Wistar (Crl:Wl(Han)) rats.

In a subchronic diet study by Van Berol et al. (2018), NOAELs of 2’-FL, manufactured using a genetically engineered *E. coli* K12 strain (manufacturer - Glycosyn and FrieslandCampina Domo), were determined to be 10% of the diet, corresponding to 7,250 mg/kg bw/d and 7,760 mg/kg bw/d for male and female Wistar Han IGS rats (Crl:WI(Han)) rats, respectively.

The findings from three unpublished studies are consistent with those found from the above-mentioned two published studies. An unpublished study by Case and Yoon (2020) reported a NOAEL of 7,500 mg/kg bw/day in rats for APTech’s 2’-FL prepared via genetically modified *C. glutamicum* APC199. An unpublished study by Penard et al. (2015) reported a NOAEL of 5,000 mg/kg bw/day in rats for Glycom's 2’-FL manufactured via a genetically engineered *E. coli* K12 strain (GRN 650, FDA 2016a). From an unpublished diet study of 2’-FL, which was prepared via a genetically modified *E. coli* BL21 strain, Jennewein Biotechnologie reported NOAELs of 7,660 mg/kg bw/day and 8,720 mg/kg bw/day in female and male rats, respectively (GRN 571, FDA, 2015).

Based on the NOAEL values reported for various preparations of 2’-FL, the Expert Panel concluded that a NOAEL of 5,000 mg/kg bw/day is an appropriate basis

for a determination of safety and that various purified 2'-FL ingredients showed similar toxicology profiles regardless of methods of manufacture.

Human Clinical Studies

Healthy infants received daily doses of up to 1.0 g of 2'-FL/L for up to 6 months (Goehring et al., 2016; Marriage et al., 2015; Nowak-Wegrzyn et al., 2019; Puccio et al., 2017; Steenhout et al., 2016; Storm et al., 2019). No adverse effects of 2'-FL were reported on the measured outcomes listed as follows: growth and tolerance (Marriage et al., 2015; Puccio et al., 2017; Storm et al., 2019), 2'-FL absorption and excretion (Marriage et al., 2015), formula intake, behavioral patterns, and morbidity including parents-reported adverse events (Puccio et al., 2017; Storm et al., 2019), global average microbial composition profile (Steenhout et al., 2016), and markers of immune functions (Goehring et al., 2016).

A human study with 100 adults evaluated the effect of 2'-FL on safety including gastrointestinal symptoms, clinical chemistry, hematology, and gut microbiota (Table 20; Elison et al., 2016). Healthy adults received 2'-FL or lacto-*N*-neotetraose (LNnT) doses up to 20 g/day, either alone or in combination, for up to 2 weeks. Volunteers taking the highest dose (20 g/day) of 2′-FL reported increased bloating, flatulence, rumbling, nausea, and diarrhea as well as loose stools and urgency to pass stools. Compared to the control group, the high dose 2'-FL or LNnT groups (20 g/day) had significantly higher Bristol Stool Form Scale (BSFS) scores, indicating softer stools although the differences were small (<0.5 points increase). However, the clinical chemistry and hematology parameters remained within the normal ranges throughout the intervention (data were not presented in this study). Thus, the authors concluded that supplementation of 2′FL at daily doses up to 20 g was shown to be safe and well tolerated. However, the Expert Panel concluded that a safe daily dose in adults was 10 g.

Overall, human clinical studies showed that various 2'-FL preparations in relatively high purity was safe in infants: formulas supplemented with 1.0 g/L 2'-FL were well tolerated in infants. In adults, daily doses of up to 10 g/day were shown to be safe.

Potential Allergenicity

It is not likely that 2'-FL would cause adverse allergenic reactions for the following reasons: 1) The protein content in the APTech's 2'-FL is ≤ 100 µg/g or 0.01% (w/w) as indicated in the specifications; 2) none of introduced gene products [GDP-L-fucose synthase (WcaG), GDP-D-mannose 4,6-dehydratase (Gmd), lactose permease (LacY), and α-1,2-fucosyltransferase (α-1,2-ft)] were found to have homology in amino acid sequences with those of allergenic proteins when the allergenic potential was

screened using the database, http://allergenonline.org/databasefasta.shtml (March 23, 2018 version); and 3) no production microorganisms and residual DNA were present in APTech's 2'-FL.

Safety of Production Microorganism

In vitro tests demonstrated that *C. glutamicum* APC199 did not produce biogenic amines and did not have hemolytic and gelatinase activities. The whole genomic sequencing found no virulence genes in *C. glutamicum* APC199. None of the introduced gene products [GDP-L-fucose synthase (WcaG), GDP-D-mannose 4,6-dehydratase (Gmd), lactose permease (LacY), and α-1,2-fucosyltransferase (α-1,2-ft)] have homology in amino acid sequences with those of allergenic proteins.

Analysis by validated quantitative polymerase chain reaction (qPCR) methods revealed that the levels of residual genes [host DNA and four foreign genes (*gmd*, *wcaG*, *α-1,2-ft*, and *lacY*)] were below the detection levels in the final 2'-FL ingredient. The detection of the genes from the vector was used as a proxy for the presence of the host organism.

General Recognition of the Safety of 2'-FL

2′-FL is a naturally occurring trisaccharide found in human milk and is, therefore, typically referred to as a human milk oligosaccharide (HMO). The FDA has issued 'no question' letters in response to several GRAS notifications related to the use of 2'-FL in infant formulas and selected conventional foods. Additionally, in all the studies summarized in previous GRAS determinations, there were no significant adverse effects/events or tolerance issues attributable to 2'-FL in either adults or infants. Because this safety evaluation was based on generally available and widely accepted data and information, it satisfies the so-called "common knowledge" element of a GRAS determination.

In addition, the intended uses of 2'-FL have been determined to be safe through scientific procedures as set forth in 21 CFR 170.3(b), thus satisfying the so-called "technical" element of the GRAS determination. The 2'-FL that is the subject of this GRAS determination is produced by a genetically engineered *Corynebacterium glutamicum* APC 199 strain, a non-pathogenic and non-toxigenic strain, and its purity is over 94%. The raw materials and processing aids used in the manufacturing process are food grade and/or commonly used in food manufacturing processes.

The literature search did not identify safety or toxicity concerns related to 2'-FL. Subchronic oral toxicity studies conducted on 2'-FL ingredients manufactured by other processes reported the NOAEL values of 2'FL as at least 5,000 mg/kg bw/day. The addition of 2'-FL at the dose of up to 2,000 mg/L was well tolerated and supported normal growth patterns in neonatal piglets. The literature also contains a wealth of publicly available studies on the safety of 2'-FL in infants and other human age groups.

APTech's 2'-FL is chemically and structurally identical to the 2'-FL found in human milk and, therefore, the safety of APTech's 2'-FL is supported by the known consumption of 2'-FL from human breast milk in infants. In addition, APTech's 2'-FL is chemically and structurally identical to synthetic 2'-FL preparation, for which FDA issued a 'no question' letter.

Conclusion

We, the undersigned members of the Expert Panel, have individually and collectively critically evaluated the materials summarized above on the safety of APTech's 2'-FL and other information deemed appropriate and unanimously conclude that APTech's 2'-FL, manufactured as described in the dossier and consistent with cGMP, and meeting appropriate food grade specifications, is Generally Recognized As Safe (GRAS) based on scientific procedures for use as an ingredient in term infant formulas at 2.4 g/L and in various conventional foods at levels specified in the accompanying dossier. It is our opinion that other qualified and competent scientists reviewing the same publicly available information would reach the same conclusions.

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[redacted]

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