From:	Susan S Cho
To:	Hice, Stephanie
Subject:	Re: GRN 000932 - Questions for Notifier
Date:	Friday, October 23, 2020 4:56:41 PM
Attachments:	GRN 932 APTech Response to FDA Questions 10-23-2020 Final submitted to FDA.pdf image001.png

Dear Dr. Hice,

Please find our response in the attached document. We hope that the information in the attached responds fully to FDA's questions . We would be happy to provide you with any fiurther information you may need. Thank you very much. have a nice weekend!

Sincerely,
Susan
Susan Cho, Ph.D.
NutraSource, Inc. (new company name, AceOne RS)
Clarksville, MD 21029 +1-410-531-3336 (O) +1-301-875-6454 (C)

On Friday, October 9, 2020, 05:20:19 PM EDT, Hice, Stephanie <stephanie.hice@fda.hhs.gov> wrote:

Dear Dr. Cho,

During our review of GRAS Notice No. 000932, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your response.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD

Staff Fellow (Biologist)

Division of Food Ingredients

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

October 23, 2020

Dr. Stephanie Hice
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: Response to FDA Questions related to GRN 932, 2'-fucosyllactose

From: Susan Cho, NutraSource, Inc. (new company name, AceOne RS)

Dear Dr. Hice,

In response to FDA questions, we have prepared our response as follows.

Regulatory

1. *Regarding the intended uses (Table 1, page 6-7):*
   - *The food category of "infant meal replacers" should be clarified. It is our understanding that the notifier intends to use this ingredient in products such as Pediasure. We note that this type of meal replacement product is intended for toddlers/ young children over 12 months of age. Meal replacement products intended for infants (i.e., children less than 1 year of age) are regulated as infant formula.*
   - *As a general comment, we note that we would consider whey-based infant formula to be subsumed under the larger category of milk-based infant formula.*

   Response

   APTech thanks the Agency for clarifying on infant meal replacers. APTech would like to clarify that the food category is intended for toddlers or young children over 12 months of age and that the term 'infant meal replacers' should be replaced with 'meal replacers for toddlers/young children over 12 months of age.'

   We agree that whey-based formula is under the larger category of milk-based formula.

2. *In section 1.C.3 (page 8) and section 6.G.1 (page 62) the notifier states: "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." Because this ingredient is intended for use in infant formula, the definition of a "nutrient" is defined in 21 CFR Part 106.3. In our view, 2'-FL does not meet the definition of a "nutrient" as defined in 21 CFR Part 106.3. For the administrative record, the notifier should clarify their statement to reflect the fact that 2'-FL is not considered a nutrient and merely a constituent in (or component of) human milk.*

Response

Thank you for the comments. We concur.

3. *Table 7 (page 23) is missing the notifier's specification for coliforms. For the administrative record, please provide an updated table.*

Response

Please see the updated Tables 5 to 7. The yellow highlights indicate amendments.

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	ISO 17294:2014 (modified)* 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)	
Difucosyllactose, %	≤ 3 (Area)	
3-Fucosyllactose, %	≤ 1 (Area)	
Fucosyl-Galactose, %	≤ 1 (Area)	
Glucose, %	≤ 3 (Area)	
Galactose, %	≤ 3 (Area)	
Fucose, %	≤ 1 (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
Enterobacteriaceae	Absent in 10 g	ISO 21528-1
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)
Residual ethanol, mg/kg	≤ 1,000	USP, 38nd Rev. – National Formulary 33th Ed., USP <467>, (2015). (modified).

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

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, %	≤ 1 (Area)	ND	ND	ND	ND	ND
Difucosyllactose, %	≤ 3 (Area)	0.24	0.86	0.58	0.02	0.02
Fucosyl-Galactose, %	≤ 1 (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, %	≤ 1 (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
Enterobacteriaceae, 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
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
Residual ethanol, mg/kg	≤ 1,000	221	220	221	229	232

Abbreviations: cfu = colony forming unit; EU =endotoxin unit; 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, %	≤ 3 (Area)	NS	≤ 1.0	≤ 5% (Area)	NS
3-FL, %	≤ 1 (Area)	NS	NS	≤ 5% (Area)	NS
Fucosyl-galactose, %	≤ 1 (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, %	≤ 1 (Area)	≤ 2%	≤ 1.0	≤ 3% (Area)	NS
Allo-lactose, %	NS	≤ 2%	NS	NS	NS
Total HMOs, %	NS	NS	≥96.0	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	ND in 10 g	ND in 10 g	ND in 10 g	ND in 11 g (w/ Coliform)	ND in 10 g
Coliform, cfu/g	≤ 10	NS	NS		NS
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
Residual ethanol, mg/kg	≤ 1,000	NS	NS	NS	NS

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

4. *The sub-header, "Absence of Host Organism, Introduced Antibiotic Resistant Genes, and Enzyme Residues" in Section 2.E. (page 24) does not include a discussion on antibiotic resistance genes or enzyme residues. For the administrative record, please explain if antibiotic resistance genes and enzyme residues are present in the production strain or are expected to be present in the final product.*

Response

The absence of microorganism and enzyme residues 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 genes from the plasmid was used as a proxy for the presence of the host organism. These methods target short subsequences of the host DNA and four inserted foreign genes encoding GDP-D-mannose-4,6- dehydratase (*gmd*), GDP-L-fucose synthase (*wcaG*), lactose permease (*lacY*), and α-1,2-fucosyltransferase (*α-1,2-ft*). The results show that the detection limit of *gmd*, *wcaG*, *α-1,2-ft*, and *lacY* was less than 6.25×10^{-6} ng/μL (6.25×10^{-3} ppb) and the 16s DNA in APC199 genome was less than 1.25×10^{-3} ng/ μL (1.25 ppb). The levels of residual genes [the host DNA and four foreign genes (*gmd*, *wcaG*, *α-1,2-ft*, and *lacY*)] were below the detection levels (Table R4.1).

Table R4.1. Levels of Residual DNA in 3 Batches of 2'-FL

Parameter	Sample number (3 non-consecutive batches)		
Residual DNA by qPCR	2'-FL-CG-013	2'-FL-CG-014	2'-FL-CG-015
gmd encoding GDP-D-mannose-4,6-dehydratase	<LOD	<LOD	<LOD
wcaG encoding GDP-L-fucose synthase	<LOD	<LOD	<LOD
α-1,2-ft encoding lactose permease	<LOD	<LOD	<LOD
lacY encoding α -1,2-fucosyltransferase	<LOD	<LOD	<LOD
16s DNA in APC199 genome	<LOD	<LOD	<LOD

2'-FL = 2'-fucosyllactose; DNA = deoxyribonucleic acid; qPCR = quantitative polymerase chain reaction; LOD = limit of detection.

Antibiotic resistance gene detection was performed using whole genome sequence information of the *C. glutamicum* ATCC13032 strain (parent strain of *C. glutamicum* APC199), and the result was matched based on the genomic database in the "Resfinder" web program. ResFinder identifies acquired antimicrobial resistance genes and/or chromosomal mutations in total or partial sequenced isolates of bacteria. ResFinder consists of two programs, ResFinder.py identifying acquired genes and PointFinder.py identifying chromosomal mutations. The software and databases are available online: https://cge.cbs.dtu.dk/services/ResFinder. Antibiotic resistance genes were shown to be absent in *C. glutamicum* ATCC13032.

However, kanamycin resistance gene is present in the production microorganism, *C. glutamicum* APC199, because this gene was inserted as a marker of *C. glutamicum* APC199 harboring pFP110. As discussed in our response to Question 19 and Appendix R.B of this document, it is not likely that *C. glutamicum* APC199 is capable of DNA transfer to other

organisms.

More importantly, the production strain *C. glutamicum* APC199 will be used for 2'-FL production purposes only in a highly contained fermentation system. After each batch fermentation is completed, the bacterial cells are completely removed by membrane filtration and heat treated. In addition, the finished 2'-FL ingredient does not have any residual production microorganism, as demonstrated by PCR analysis (please see Appendix G or pages 110 to 112 of the original submission).

5. *On page 34 of the notice, the notifier states, "The proposed use levels are similar to those described in another 2'-FL GRAS notice" but does not provide a reference for the notice. For the administrative record, please provide a reference for the GRAS notice described.*

Response

GRN 735 is the reference. It now reads as follows:

'The proposed use levels are similar to those described in another 2'-FL GRAS notice (GRN 735).'

6. *On page 73 of the notice, the notifier states, "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". We note that it is the uses of C. glutamicum-produced ingredients in conventional foods that have been concluded to be GRAS, not the production microorganism itself.*

Response

We concur.

Chemistry

7. *In the method of manufacture description (page 16), the notifier notes two activated carbon treatment steps. Is the second step optional?*

Response

The second activated carbon treatment is optional. The first step is essential for color removal.

8. *Based on results of batch analyses (Table 6, page 20), the protein content of 2'-FL (<10 µg protein per kg 2'-FL) is well below the stated specification of ≤100 µg/g (≤100 mg/kg). We request that the notifier lower this specification to better reflect the method of manufacture and resulting composition of the ingredient or provide a discussion of why the higher limit is needed.*

Response

Although the batch analysis showed less than 100 µg/g (or 100 mg/kg), APTech would like to keep the current specification for protein, if possible, to be in line with protein

specifications of other GRAS notices, which received FDA's 'no question' letters.

9. *Based on results (Table 6, page 20) of batch analyses for arsenic (<0.01 mg/kg), we request that the notifier lower the arsenic specification of NMT 0.1 mg/kg or provide a discussion of why the higher limit is needed.*

   Response

   Although the batch analysis showed less than 0.01 mg/kg, APTech would like to keep the current specification for arsenic (As) to be in line with As specifications of other GRAS notices, which received FDA's 'no question' letters.

10. *Please indicate if the notifier has established a limit for residual solvent (ethanol) used for crystallization.*

    Response

    The ethanol analysis of 5 batches showed an average of 223 ppm, which is lower than the limit of the class 3 residual solvent classified in USP 467. We want to add residual ethanol specifications of less than 1,000 mg/kg. Please see the revised Tables 5 to 7 presented on pages 2 to 7 of this document.

11. *While it appears that the notifier has set specifications for individual carbohydrates to match those of GRN 000571, the limits suggest more variation in the composition of 2'- FL than is indicated by batch analyses or the high percentage (NLT 94%) of the main component, 2'-FL.*

    - *Specifications for lactose and difucosyllactose (DFL) are set to NMT 5% each. Batch analyses show 0.09-0.11% lactose and 0.02-0.86% DFL. Please address if these ranges are typical, or alternatively, if large variation is expected, and adjust limits accordingly.*
    - *Specifications for 3-fucosyllactose (3-FL), fucosyl-galactose (FG), and fucose are NMT 3% (FG, fucose) and NMT 5% (3-FL). Based on batch analyses, none of these carbohydrates are detected in the 2'-FL ingredient. They are also not included in the summary of "other components" on page 25. The notifier should clarify if they expect these minor components based on their method of manufacture and adjust limits accordingly. If the aforementioned components are present, please include them in the page 25 discussion.*

    Response

    APTech has lowered the specifications to 3% for difucosyllactose (DFL), and 1% for 3-fucosyllactose (3-FL), fucosyl-galactose (FG), and fucose. Please see the revised Tables 5 and 7 presented on pages 2 and 6 of this document.

12. *The method of analyses indicated for metals (Table 5, page 19) include methods for determination of mercury (IEC 62321-4; 2014) and of cadmium, lead, and chromium (IEC 62321-5; 2014) in polymers, metals, and electronics by atomic absorption spectrometry (AAS), atomic fluorescence spectrometry (AFS), inductively coupled plasma (ICP) optical emission spectrometry (OES), and ICP-mass spectrometry (ICP-*

MS). An additional method is also cited for analysis of water by ICP-MS. Please clarify the method of detection for each metal and confirm that the methods are validated for the intended purpose.

Response

The heavy metal analysis was conducted by the Korea Institute of Ceramic Engineering & Technology (KICET) using inductively coupled plasma mass spectrometry (ICP-MS; ELAN DRC II equipment, Perkin-Elmer Inc., USA). The ICP-MS method of metal analysis (mercury, cadmium, lead, and arsenic) was based on ISO 17294:2014 with modifications for sample preparation steps. Each metal analysis has been validated.

The limits of quantification (LOQ) are 0.01 mg/kg for arsenic, lead, and mercury and 0.008 mg/kg for cadmium. The analysis results of 5 batches showed that concentration of each metal was below the LOQ.

13. *Other oligosaccharides are noted in the safety narrative for 2'-FL. Does the intended use of 2'-FL include use in formulas that contain other oligosaccharides? Please address, including examples as warranted.*

Response

APTech does not intend to combine 2'-FL with other sources of poorly digested carbohydrates at this time. However, APTech is a manufacturer of 2'-FL ingredient, not a manufacturer of infant formula and other foods. We recognize that other oligosaccharides, such as galacto-oligosaccharide (GOS) and fructo-oligosaccharide (FOS), are often used as ingredients in infant formulas. In addition, other human milk oligosaccharides (HMOs), such as lacto-N-neotetraose (LNnT), DFL, 3'-sialyllactose (3'-SL), and 6'-sialyllactose (6'-SL), may be used. Possible side effects of 2'-FL and other indigestible carbohydrates may be related to gastrointestinal tolerance. However, the use level of APTech's 2'-FL, up to 2,400 mg/L, represents an approximate average concentration of 2'-FL present in human milk. Even if 2'-FL is used in combination with other oligosaccharides, it is not expected to have gastrointestinal tolerance issue as long as other oligosaccharides are used within the levels of oligosaccharides found in human milk, which is inherently well tolerated.

For other oligosaccharides, for which target levels cannot be established based on natural occurrence, safe use levels can be established based on tolerance study outcomes.

Human clinical studies showed that the combined use of 2'-FL with other HMOs, GOS, or FOS did not result in adverse effects on the measure outcomes including gastrointestinal tolerance, adverse events, growth, and/or microbial composition profile in term infants. As shown in Table 18 of the original submission (page 57), examples include the test formula containing 1.0 g 2'-FL/L and 0.5 g LNnT/L (Nowak-Wegrzyn et al., 2019), 1.0 g/L 2'-FL plus 0.5 g/L LNnT (Puccio et al., 2017), and 2.2 g/L GOS plus 0.2 g/L 2'-FL or 1.4 g/L GOS plus 1.0 g/L 2'-FL (Marrige et al., 2015). A recent human clinical study by Berger et al. (2016, 2020) also demonstrated that the formula containing 1.0 g/L 2'-FL plus 0.5 g/L LNnT was well tolerated with no side effects on fecal microflora and antibiotic requirements in term infants. Kajzer et al. (2016; cited in Reverri et al., 2018) also reported that the combined use of 2'-FL with short chain FOS (scFOS) (0.2 g/L 2'-FL plus 0.2 g/L scFOS) was well tolerated in healthy term infants.

In order to comply with Section 412(d)(1) of the Food, Drug and Cosmetic Act, manufacturers of infant formula must notify FDA to provide a basis for the safety of the

formulation prior to introducing a new formulation to the marketplace. Thus, it is not likely that a manufacturer planning to use 2'-FL and oligosaccharides in excess of the intended use levels of 2'-FL specified in this GRAS notice would proceed before obtaining the data supporting the safety and tolerability of the target level.

In addition, the use of other HMOs, such as DFL, LNnT, 3'-SL sodium salt, 6'-SL sodium salt, GOS, and FOS, have been the subjects of GRAS notices for which FDA had no questions on the safety of intended uses, and the regulatory status of such oligosaccharides are summarized below. There is no apparent new risk posed by the APTech ingredient that has not already been considered in the previous GRAS notices.

Regulatory Status of Other Oligosaccharides

GRN No (date of closure)	Substance	Intended Food Uses (The numbers in parenthesis indicate the maximum use levels)	Company
Human Milk Oligosaccharides			
815 (8/20/2020)	A mixture of 2'-FL and difucosylllactose (DFL; approximate ratio = 8:1)	Formula 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 A/S
547 (10/2/2015)	Lacto-N-neotetraose	Formula for term infants (up to 0.6 g/L); other selected foods and drinks (0.02-1.2 g/serving)	Glycom A/S
659 (11/23/2016)	Lacto-N-neotetraose	Formula for term infants (up to 600 mg/L); other selected foods and drinks (0.02-3 g/serving)	Glycom A/S
766 (9/26/2018)	3'-sialyllactose sodium salt (3'-SL)	Formula for infants (up to 238 mg 3'-SL sodium salt or 230 mg 3'-SL/L); other foods and beverages (24.8-3,104 3'-SL sodium salt or 24-3,000 mg 3'-SL/serving)	GeneChem, Inc.
880 (4/13/2020)	3'-SL sodium salt	Formula for term infants (up to 0.2 g/L); beverages and formula for young children (0.15 g/L); foods for infants and young children (1.25 g/kg); other selected foods and beverages (2.5 g/kg or 0.25-0.5 g/L)	Glycom A/S
881 (4/13/2020)	6'-siallactose sodium salt (6'-SL)	Formula for term infants (up to 0.4 g/L); beverages and formula for young children (0.3 g/L); foods for infants and young children (2.5 g/kg); other selected foods and beverages (5-10 g/kg or 0.5-1 g/L)	Glycom A/S
Galacto-oligosacchiarides (GOS)			
233 (9/4/2009)	Combination of GOS and polydextrose	Formula for term infants (up to 2 g/L GOS and 2 g/L polydextrose)	Mead Johnson & Company

236 (7/28/2008)	GOS	Formula for term infants (5 g/L); other selected foods and drinks (1.0-7.5 g/serving)	Friesland Foods Domo
286 (9/4/2009)	GOS	Formula for term infant (7.2 g/L)	GTC Nutrition
334 (10/27/2010)	GOS	Formula for term infants (7.2 g/L); other selected foods and drinks (0.3-9.5 g/serving)	Yakult Pharmaceutical Industry Co., Ltd.
489 (5/22/2014)	GOS	Formula for infants (up to 4 g/L); other selected foods and drinks (up to 3.8 mg/g or 2.66 g/serving)	International Dairy Ingredients, Inc.
495 (5/30/2014)	GOS	Formula for term infants (up to 7.2 g/L)	Clasado Inc.
518 (12/22/2014)	GOS	Selected foods and drinks (0.3-11 g/serving)	New Francisco Biotechnology Corporation
569 11/25/ 2015	GOS	Formula for term infants (up to 7.2 g/L)	New Francisco Biotechnology Corporation
620 7/21/ 2016	GOS	Formula for term infants (up to 7.8 g/L)	Nestle Nutrition
721 (12/19/2017)	GOS	Formula for term infants (up to 7.2 g/L); other selected foods and drinks (up to 33.4%)	Vitalus Nutrition Inc.
729 (3/27/2018)	GOS	Formula for tern infants (up to 7.8 g/L); other selected foods and drinks (up to 11 g/serving)	Neo Cremar, Co., Ltd.
Fructo-oligosaccharides (FOS) and Inulin-based Oligofructose			
392 (5/7/2012)	Oligofructose	Formula for term infant (up to 3 g/L)	Pfizer Nutrition and BENEO-Orafti
537 (2/6/2015)	Short-chain FOS	Formula for term infants (up to 500 mg/100 mL)	Ingredion Incorporated
576 (12/9/2015)	Oligofructose and inulin	Formula for term infants (6.12 g/L oligofructose and 0.68 g/L inulin)	Nutricia North America, Inc.
797 (11/15/ 2018)	FOS	Formula for term infants (up to 500 mg/100 mL)	New Francisco (Yunfu City) Biotechnology Corporation

References

Berger PK, Plows JF, Jones RB, Alderete TL, Yonemitsu C, Poulsen M, Ryoo JH, Peterson BS, Bode L, Goran MI. Human milk oligosaccharide 2'-fucosyllactose links feedings at 1 month to cognitive development at 24 months in infants of normal and overweight mothers. PLoS One. 2020;15(2):e0228323.

Berger B, Sprenger N, Grathwohl D, Alliet P, Puccio G, Steenhout P, Lausanne NSA. Stool microbiota in term infants fed formula supplemented with synthetic human milk oligosaccharides is associated with reduced likelihood of medication. J Pediatric Gastroenterol Nutr. Abstract 1190. J Pediatr Gastroenterol Nutr. 2016;63 Suppl 2:S406.

FDA, 2009. GRN 233, Combination of galacto-oligosaccharides and polydextrose, filed by Mead Johnson & Company. Date of closure: Sep 4, 2009. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=233

FDA, 2008. GRN 236, Galacto-oligosaccharides, filed by Friesland Foods Domo. Date of closure: Jul 28, 2008. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=236

FDA, 2009. GRN 286, Galacto-oligosaccharides, filed by GTC Nutrition. Date of closure: Sep 4, 2009. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=286

FDA, 2010. GRN 334, Galacto-oligosaccharides, filed by Yakult Pharmaceutical Industry Co., Ltd. Date of closure: May 3, 2010. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=334

FDA, 2012. GRN 392, Oligofructose, filed by Pfizer Nutrition and BENEO-Orafti. Date of closure: May 7, 2012. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=392

FDA, 2014. GRN 489, Galacto-oligosaccharides, filed by International Dairy Ingredients, Inc. Date of closure: May 22, 2014. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=489

FDA, 2014. GRN 495, Galacto-oligosaccharides, filed by Clasado Inc. Date of closure: May 30, 2014. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=495

FDA, 2014. GRN 518, Galacto-oligosaccharides, filed by New Francisco Biotechnology Corporation. Date of closure: Dec 22, 2014. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=518

FDA, 2015. GRN 537, Short-chain fructo-oligosaccharides, filed by Ingredion Incorporated. Date of closure: Feb 6, 2015. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=537

FDA, 2015. GRN 547, Lacto-N-neotetraose, filed by Glycom A/S. Date of closure: Oct 2, 2015. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=547

FDA, 2015. GRN 569, Galacto-oligosaccharides (GOS), filed by New Francisco Biotechnology Corporation. Date of closure: Nov 25, 2015. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=569

FDA, 2015. GRN 571, 2'-Fucosyllactose, filed by Jennewein Biotechnologie, GmgH. Date of closure: Nov 6, 2015. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571

FDA, 2015. GRN 576, Oligofructose and inulin, filed by Nutricia North America, Inc. Date of closure: Dec 9, 2015. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=576

FDA, 2016. GRN 620, Galacto-oligosaccharides, filed by Nestle Nutrition. Date of closure: Jul 21, 2016. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=620

FDA, 2016. GRN 659, Lacto-N-neotetraose, filed by Glycom A/S. Date of closure: Nov 23, 2016. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=659&sort=GRN_No&order=DESC&startrow=1&type=basic&search=neotetraose

FDA, 2017. GRN 721, Galacto-oligosaccharides, filed by Vitalus Nutrition Inc. Date of closure: Dec 19, 2017. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=721

FDA, 2018. GRN 729, Galacto-oligosaccharides, filed by Neo Cremar, Co., Ltd. Date of closure: Mar 27, 2018. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=729

FDA, 2018. GRN 766, 3'-sialyllactose sodium salt, filed by GeneChem, Inc. Date of closure: Sep 26, 2018. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=766

FDA, 2018. GRN 797, Fructooligosaccharides, filed by New Francisco (Yunfu City) Biotechnology Corporatiom. Date of closure: Nov 15, 2018. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=797

FDA, 2019. GRN 815, 2'-fucosyllactose and difucosyllactose, filed by Glycom A/S. Date of closure: Aug 20, 2019. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=815

FDA, 2020. GRN 880, 3'-sialyllacotse sodium salt, filed by Glycom A/S. Date of closure: Apr 13, 2020. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=880

FDA, 2020. GRN 881, 6'-sialyllactose sodium salt, filed by Glycom A/S. Date of closure: Apr 13, 2020. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=881

Kajzer J, Oliver J, Marriage B. Gastrointestinal tolerance of formula supplemented with oligosaccharides.FASEB J. 2016; 30: 671.

Marriage BJ, Buck RH, Goehring KC, Oliver JS, Williams JA. Infants fed a lower calorie formula with 2'FL show growth and 2'FL uptake like breast-fed infants. J Pediatr Gastroenterol Nutr. 2015;61:649-58.

Nowak-Wegrzyn A, Czerkies L, Reyes K, Collins B, Heine RG. Confirmed hypoallergenicity of a novel whey-based extensively hydrolyzed infant formula containing two human milk oligosaccharides. Nutrients. 2019;11(7). pii: E1447.

Puccio G, Alliet P, Cajozzo C, Janssens E, Corsello G, Sprenger N, Wernimont S, Egli D, Gosoniu L, Steenhout P. Effects of infant formula with human milk oligosaccharides on growth and morbidity: A randomized multicenter trial. J Pediatr Gastroenterol Nutr. 2017;64:624-31.

Reverri EJ, Devitt AA, Kajzer JA, Baggs GE, Borschel MW. Review of the clinical experiences of feeding infants formula containing the human milk oligosaccharide 2′fucosyllactose. Nutrients. 2018; 10(10):1346.

Microbiology

14. For the administrative record, please provide a detailed description of the production strain including phenotypic (e.g., production of antibiotics) and genotypic characteristics (e.g., introduced and excised genes).

Response

The production strain of APTech's 2'-FL is *Corynebacterium glutamicum* APC199. We have described APC 199 in Appendix B, Production Strain Construction (page 73 of the original submission). Compared to *E. coli* based 2'-FL production, we have not performed any gene excision of the host strain because *C. glutamicum* ATCC13032 does not have beta-galactosidase activity, which hydrolyze lactose. On the other hand, *E. coli* K12 has beta-galactosidase activity, and the genes encoding beta-galactosidase should be excised for successful production of 2'-FL. We have introduced *lacY* (lactose permease) to the plasmid for uptake of lactose into the cell. Two more genes in the pathway of GDP-L-fucose synthesis (*gmd* and *wcaG*) and *a-1,2-ft* (alpha-1,2-fucosyltransferase, which transfers the fucose moiety of GDP-L-fucose to lactose to produce 2'-FL) were introduced into the same plasmid (pFP110).

The genotype of APC199, pFP110 plasmid harboring the wild-type *C. glutamicum* ATCC13032, is *C. glutamicum* ATCC13032/pFP110 [ptuf-a-1,2-ft-gmd-wcaG-lacY, nptII].

The host strain, *Corynebacterium glutamicum*, is available from ATCC as 13032 and Korea Collection for Type Cultures (KCTC) as 1445. The *C. glutamicum* strain is gram positive, nonmotile, and ellipsoidal spheres to short rods. *C. glutamicum* ATCC13032 is the representative wild-type strain, traditionally well-known as an excellent producer of various amino acids, and has a long history of use in the food industry. *C. glutamicum* was discovered in the 1950's in Japan as a natural producer of glutamic acid (Kinosha et al., 1957). The whole genomic sequencing of this strain has been completed (GenBank: NC_006958.1; Kalinowski

et al., 2003).

C. glutamicum ATCC13032 is non-pathogenic and non-toxigenic, and is classified as a risk group 1 by German Technische Regel für Biologische Arbeitsstoffe (TRBA; https://www.atcc.org/products/all/13032.aspx). This means that the microorganism is not known to consistently cause disease in healthy adult humans and has minimal potential hazard to laboratory personnel and the environment.

There are no reports of *C. glutamicum* being a pathogen or antibiotics producer. Although various soil microorganisms producing antibiotics have been found and reported, there are no reports that *C. glutamicum* produces antibiotics (Chandra and Kumar, 2017).

References

Chandra N, Kumar S. 2017. Antibiotics Producing Soil Microorganisms. In: Hashmi M., Strezov V., Varma A. (eds) Antibiotics and Antibiotics Resistance Genes in Soils. Soil Biology, vol 51. Springer, Cham. https://doi.org/10.1007/978-3-319-66260-2_1.

Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Krämer R, Linke B, McHardy AC, Meyer F, Möckel B, Pfefferle W, Pühler A, Rey DA, Rückert C, Rupp O, Sahm H, Wendisch VF, Wiegräbe I, Tauch A. The complete Corynebacterium glutamicum ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J Biotechnol. 2003 4;104(1-3):5-25.

Kinoshita S, Udaka S, Shimono M. Studies on the amino acid fermentation. Part 1. Production of L-glutamic acid by various microorganisms. J Gen Appl Microbiol. 1957;3 (3):193–205.

15. *Please describe the origin of the donor genes (e.g., are they de novo synthesized or of bacterial origin).*

Response

The four foreign genes introduced in the plasmid pFP110 were synthesized using GenBank sequence information. The genes *gmd* (GenBank: AAC75114 .1, coding for a GDP-mannose 4,6 dehydratase), *wcaG* (GenBank: AAC75113.1, coding for a GDP-L-fucose synthase), and *lacY* (GenBank: AAC 73446.1, coding for a lactose permease) have been derived from *E. coli* ATCC 700926 sequence. The gene *α-1,2-ft* (GenBank: ADY53338.1, coding for a α -1,2-fucosyltransferase) have been derived from the *Pseudopedobacter saltans* ATCC 51119 sequence information. The origin of the donor genes is described on page 13 of the original submission.

Page 13 of the original submission

'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

16. *In Appendix B (pages 73-77), the notifier states that "Heterologous genes necessary to biosynthesize 2'-FL in Corynebacterium glutamicum were inserted into the pCN01 plasmid, resulting in the construction of the pFP110 plasmid"; according to the information presented in the notice, the pCN01 plasmid and the pFP110 plasmid contain a kanamycin resistance gene. In Table B.2., the notifier states that the function of the kanamycin resistance gene is "…for antibiotics selection" (page 77). Please include an explanation of what this statement means. Furthermore, please describe the antibiotic resistance profile of the production strain.*

Response

What we tried to describe is as follows:
Although the pFP110 plasmid has the kanamycin resistance gene (*nptII*), it was used as a selection marker during the construction of the plasmid pFP110 and the selection of production strain *C. glutamicum* APC199. The gene is not utilized at any stage in the 2'-FL production, and kanamycin is not added in any stages of fermentation.

Detection of antibiotic resistant gene in the host strain

Antibiotic resistance gene detection was performed using the whole genome sequence information of the *C. glutamicum* ATCC13032, and the result was matched based on the genomic database in the "Resfinder" web program. ResFinder identifies acquired antimicrobial resistance genes and/or chromosomal mutations in total or partial sequenced isolates of bacteria. ResFinder consists of two programs, ResFinder.py identifying acquired genes and PointFinder.py identifying chromosomal mutations. The software and databases are available online: https://cge.cbs.dtu.dk/services/ResFinder. No antibiotic resistance genes were detected in *C. glutamicum* ATCC13032.

In vitro evaluation of antibiotic resistance

Agar dilution method was used to evaluate the minimal inhibitory concentration (MIC) of antibiotics. *C. glutamicum* APC199 was higher than the break point MIC value regarding kanamycin (Table R16.1). It is expected since the kanamycin gene was inserted into the

plasmid DNA pFP110 for selection. In addition, similar tendency was monitored from the results conducted according to the E-test method (Table R16.2).

As shown in Tables R16-1 and R16-2, *C. glutamicum* APC199 was susceptible to various antibiotics, such as ampicillin, erythromycin, gentamicin, tetracycline, streptomycin, vancomycin, chloramphenicol, kanamycin, and clindamycin, with the exception of kanamycin. This was expected because the kanamycin resistance gene was inserted as a vector into the microorganism. Except for kanamycin, *C. glutamicum* APC199 was considered susceptible to antibiotics under the test conditions.

Table R16.1. Susceptibility of Production Microorganism to Antibiotics by Agar Dilution Method

Strain	Minimum inhibitory concentration (mg/L) of antibiotics								
	Amp	Ery	Gen	Tet	Str	Van	Chl	Kan	Cli
C. glutamicum APC199	0.5	0.5	0.25	0.125	2	0.25	4	128	2
C. glutamicum ATCC13032	0.5	1	0.125	0.125	2	0.25	4	0.25	2
EFSA break point	1	1	4	2	8	4	4	16	4

Amp = ampicillin; Chl = chloramphenicol; Cli = clindamycin; Ery = erythromycin; Gen = gentamicin; Kan = kanamycin; Str = streptomycin; Tet = tetracycline; Van = vancomycin.

Table R16.2. Susceptibility of Production Microorganism to Antibiotics by E-Test Method

Strain	Minimum inhibitory concentration (mg/L) of antibiotics							
	Amp	Ery	Gen	Tet	Str	Van	Kan	Cli
C. glutamicum APC199	1	0.19	0.06	0.64	0.5	0.125	96	1
C. glutamicum ATCC13032	0.125	0.5	0.06	0.125	0.38	0.25	0.094	1
EFSA break point	1	1	4	2	8	4	16	4

Amp = ampicillin; Chl = chloramphenicol; Cli = clindamycin; Ery = erythromycin; Gen = gentamicin; Kan = kanamycin; Str = streptomycin; Tet = tetracycline; Van = vancomycin.

17. *Please specify how the purity of the production strain is ensured during manufacturing.*

Response

During the fermentation process, including seed culture, APTech checked the purity of the culture strains by microscopic observation and/or agar plating. APTech's 2'-FL production facility is certified for Food Safety System Certification 22000 (FSSC 22000). The FSSC 22000 plan for manufacturing also includes in-process controls to minimize the amount of potential impurities to the lowest level technically possible. APTech's production process (including all processing aids, raw materials, unit operations, and filter aids) and the food safety management system comply with the following standards and certifications. In addition, APTech incorporates sterile filtration units throughout the manufacturing process.

In addition, APTech had conducted performance qualification (PQ), and media fill test in the fermenters by a sterilization protocol for 5 days with the same fermentation conditions (ex.

same media compositions, pH, aeration, agitation, and temperature). During the 5-day media fill test for repeated times, no contamination was detected in the fermentation broth.

18. *Please state whether any of the raw materials used in the fermentation are major allergens or derived from major allergens. If any of the raw materials used are major allergens or derived from major allergens, please discuss why these materials do not pose a safety concern.*

Response

No raw materials used in the fermentation are major allergens. Although lactose is derived from milk, a concentration of residual lactose in the finished ingredient is not significant.

19. *Please state whether the production strain is capable of DNA transfer to other organisms.*

Response

As described in a report issued by Korea Institute of Bioscience and Biotechnology (KRIBB), it is not likely that *C. glutamicum* APC199 is capable of DNA transfer to other organisms. Details are presented in Appendix R.B of this document, 'Evaluation of horizontal gene transfer frequency of *Corynebacterium glutamicum* APC199 by conjugation with twenty microorganisms.'

Briefly, this study was performed to evaluate the horizontal gene transfer capability of *C. glutamicum* APC199 to transfer antibiotic resistance genes to other bacteria because the strain of *C. glutamicum* APC199, a transformant of *C. glutamicum* ATCC13032 with the pFP110 plasmid, was develop for the fermentative production of 2'-FL. As part of the overall safety assessment of bacteria used to produce food ingredients, one of the standard protocols is to determine if they are capable of allowing horizontal transfer of antibiotics genes. Whole genome sequencing (see our response to Question 16) of *C. glutamicum* APC199 and ATCC13032 strains did not find antibiotic resistance genes associated with the bacterium's chromosome. Antibiotic resistance gene was limited to the kanamycin resistance associated with the *nptII* gene on the pFP110 plasmid. This was used to estimate the conjugation-mediated horizontal gene transfer potential from *C. glutamicum* APC199 to other bacteria.

Plasmid DNA reception by twenty microorganisms (commonly used, related, commonly found microorganisms found in soil or water, and potentially pathogenic microorganisms) was examined using filter-mating method. To discriminate donor microorganism (*C. glutamicum* APC199), which has kanamycin resistance associated with the *nptII* gene, from recipient microorganisms (twenty tested microorganisms), the *rpoB* gene of recipient microorganisms was mutated by the treatment of mutagen ethyl methanesulfonate (EMS) to give resistance to rifampicin. Transfer frequencies by conjugation were evaluated by the calculation of colony counting of recipient microorganisms showing dual antibiotic resistance (kanamycin and rifampicin). The gene transfer frequencies by conjugation among the twenty tested microorganisms were below or close to the detection limit of 1.1×10^{-10}; no dual antibiotic resistant microorganisms were observed among the twenty tested strains (Table 2 of Appendix R.B). These rates are about ten times lower than the minimum transfer

frequencies of 1.3 x 10^{-9} found from the literature (Table 2 of Appendix R.B). The frequencies of dual antibiotic resistance observed are more consistent with the development of spontaneous mutation (Nyinoh, 2019; O'Neill et al., 2001). There would be substantially higher rates if target horizontal transfer was occurring. Thus, KRIBB concluded that there is a very low likelihood of plasmid transfer because the transfer frequencies found in this study were consistent with spontaneous mutations instead of conjugal plasmid transfer.

More importantly, the production strain *C. glutamicum* APC199 will be used for 2'-FL production purposes only in a highly contained fermentation system. After each batch fermentation is completed, the bacterial cells are completely removed by membrane filter separation, followed by heat treatment.

In addition, the finished 2'-FL ingredient does not have any residual production microorganism, as demonstrated by PCR analysis (please see Appendix G or pages 110 to 112 of the original submission).

20. *Please state whether the fermentation process is conducted in a contained, sterile environment.*

Response
Fermentation is done in a sterile environment.

21. *On page 18 of the notice, the notifier states, "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 include these two parameters in their specifications".*

    a. *It is unclear why there is not a specification for Enterobacteriaceae, while showing in Table 7 (p. 22-23) that all other 2'-FL notifications (GRNs 000546, 000571, 000650, 000735) have provided limits for Enterobacteriaceae.*

    b. *The certificates of analysis (pages 92-96) include aflatoxin M1. For the administrative record, please clarify this discrepancy.*

Response

a. APTech has added *Enterobacteriaceae* to the specifications. Please see the revised Tables 5 to 7 for revised specifications and analytical values presented on pages 2 to 7 of this document.

b. We notice that aflatoxin M1, a metabolite of aflatoxin B1, can be found in milk or milk products obtained from livestock that have ingested contaminated feed. We wanted to confirm that no aflatoxin M1 is found in APTech's 2'-FL ingredient. However, we do not believe that aflatoxin M1 does not have to be part of the specifications because aflatoxin M1 is not expected to be produced by a fermentation process using lactose as a starting material.

22. *The notifier states that the method used to detect Cronobacter spp. is ISO/TS 22964; IDF/RM 210:2006 (page 19, 97-104). We note that this method has been revised and replaced by ISO 22964:2017, which corresponds to Microbiology of the Food Chain – Horizontal Method for*

the Detection of Cronobacter spp. Please make a statement that corrects this reference.

Response

We have contacted Eurofins USA, which conducted the *Cronobacter (Enterobacter) sakazakii* analysis. They confirmed that the analysis was done by the ISO/TS 22964|IDF/RM 210:2006 method. This method is described as a specific method for the detection of *Enterobacter sakazakii* in milk powder and powdered infant formula. The revised ISO 22964:2017 extends to the detection of *Cronobacter* spp. in food product for humans and feeding animals and environmental samples.

This method (ISO/TS 22964:2006) is also specified in the other 2'-FL GRAS notices (Glycom GRN 650 and BASF SE GRN 852) that received FDA's 'no question' letters.

23. *On page 75 of the notice, the notifier states, "The sources of the four heterologous genes are all biosafety level I microorganisms (E. coli K-12 and Pseudopedobacter saltans)". Biosafety relates to the biocontainment of an organism during laboratory work and has no relevance to our safety evaluation. For the administrative record, please briefly describe the phenotypic (e.g., pathogenicity and toxigenicity) characteristics of Escherichia coli K-12, E. coli ATCC 700926 and P. saltans ATCC 51119.*

Response

E. coli K12

E. coli K-12 is not considered a human or animal pathogen and is not toxicogenic. The National Institutes of Health (NIH) considers *E. coli* K-12 to be an organism that does not present a significant risk to health or the environment (NIH, 2019). The non-pathogenicity and non-toxigenicity of this strain were described in GRN749 and GRN899. *E. coli* K-12 and other gram negative bacteria possess lipopolysaccharides (LPS, also known as endotoxin), complex glycolipids of high molecular weight, in their cell membrane (Wassenaar and Zimmermann, 2018). Bacterial LPS has been associated with a number of diseases, including liver damage, neurological degeneration, chronic inflammation of the gut, and diabetes. When LPS enters the blood stream, this will generate a fever in animals via activation of an immunological response involving factors in the blood (complement and Toll-like receptors) that initiate the production of prostaglandins and send signals to the brain to increase body temperature (Wassenaar and Zimmermann, 2018). Although presence of LPS is almost universal in *E. coli* on isolation, not all elicit equally adverse immunostimulant reactions. For instance, LPS from non-pathogenic *E. coli* K-12 lacks O-antigen epitopes that are characteristic of pathogenic strains of *E. coli* (Kuhnert et al., 1995). In addition, K-12 strains are unable to colonize the human gut (Smith, 1975).

The wild-type strain of *E. coli* K-12 was isolated from the feces of a convalescent diphtheria patient in 1922 at Stanford University, and subcultures and derivatives of this strain were first reported in 1944 (Kuhnert et al., 1995). Since then, there has been no reference to the strain having carried an O-antigen and no *E. coli* K-12 associated case of disease has ever been reported (Kuhnert et al., 1995).

Escherichia coli K-12 strain ATCC 700926 (MG1655)

E. coli strain K12 MG1655 is a non-recombinant strain available from the American Type Culture Collection (ATCC70926) and from the Coli Genetic Stock Center as CGSC#7740. This

strain is a common laboratory strain and has been is fully sequenced (GenBank U00096.2). *E. coli* strain K12 ATCC 700926 is non-pathogenic and non-toxigenic. *Escherichia coli* ATCC 700926 (MG1655) is representative of the *E. coli* K-12 strain and is derived from the parent strain W1485 by ultraviolet light and acridine orange. *E. coli* K-12 ATCC 700926 is minimally genetically modified and cured of the temperate bacteriophage lambda and F plasmid by ultraviolet light and acridine orange, respectively (Blattner et al., 1997). Because F plasmid is associated with facilitating conjugal transfer, it is not likely that *E. coli* K-12 700926 is capable of DNA transfer to other organisms. *E. coli* K-12 lacks O antigen (Kuhnert et al., 1995; Liu and Reeves, 1994) that are characteristic of pathogenic strains of *E. coli.* Injection of *E. coli* strain, whose genome closely resembles *E. coli* K12 ATCC700926, into the bloodstream of mice did not result in any deleterious effects (Beimfohr, 2016; Kocijancic et al., 2016).

The taxonomy of *Escherichia coli* K-12 strain ATCC 700926 is shown below.

The taxonomy of *E. coli*:

(a) Domain: Bacteria

(b) Kingdom: Eubacteria

(c) Phylum: Proteobacteria

(d) Class: Gammaproteobacteria

(e) Order: Enterobacteriales

(f) Family: Enterobacteriaceae

(g) Genus: *Escherichia*

(h) Species: *Escherichia coli*

(i) Strain: *Escherichia coli* K12 or ATCC 700926

<u>*Pseudopedobacter saltans* ATCC51119</u>

Pedobacter saltans was reclassified as *Pseudopedobacter saltans* by Cao et al. (2014). *Pseudopedobacter saltans* ATCC51119 was isolated from soil in Iceland and originally classified as *Pedobacter saltans* (Steyn et al., 1998). *P. saltans* has a peculiar gliding, dancing motility and can be distinguished from other *Pedobacter strains* by their ability to utilize glycerol and the inability to assimilate D-cellobiose. The cells of *P. saltans* is gram negative, non-spore forming, strictly aerobic, and chemoorganotrophic. *P. saltans* is short rods (0.5 x 0.8-1.0 µm) with rounded or slightly tapering ends. Colonies on modified TSA are smooth, light yellow to yellow, translucent, round, 2-5 mm in diameter, and convex to slightly umbonate with entire margins. On nutrient agar, colonies are smooth, yellow, round, 2-4 mm in diameter, and convex with entire to scalloped margins. The temperature range for growth is normally between 5 and 30°C (Steyn et al., 1998). The complete genome of this strain has been sequenced (GenBank CP002545.1; Kalinowski et al., 2003).

Pseudopedobacter saltans ATCC51119 is non-pathogenic and non-toxigenic, and is classified as risk group 1 by German Technische Regel für Biologische Arbeitsstoffe (TRBA https://www.dsmz.de/microorganisms/wink_pdf/DSM20300.pdf). The taxonomy of *P. saltans* ATCC51119 is shown below.

The taxonomy of *P. saltans* ATCC51119

(a) Domain: Bacteria
(b) Kingdom: Eubacteria
(c) Phylum: Bacteroidetes
(d) Class: Sphingobacteria
(e) Order: Sphingobacteriales
(f) Family: Sphingobacteriaeae
(g) Genus: *Pseudopedobacter*
(h) Species: *Pseudopedobacter saltans*
(i) Strain: *Pseudopedobacter saltans* ATCC51119

References

Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y. The complete genome sequence of *Escherichia coli* K-12. Science. 1997;277(5331):1453-62.

Beimfohr C. A Review of research conducted with probiotic *E. coli* marketed as Symbioflor. Int J Bacteriol. 2016;2016:3535621.

Cao J, Lai Q, Li G, Shao Z. *Pseudopedobacter beijingensis* gen. nov., sp. nov., isolated from coking wastewater activated sludge, and reclassification of *Pedobacter saltans* as *Pseudopedobacter saltans* comb. nov. Int J Syst Evol Microbiol. 2014;64(Pt 6):1853-8.

Kalinowski J, Bathe B, Bartels D, Bischoff N, Bott M, Burkovski A, Dusch N, Eggeling L, Eikmanns BJ, Gaigalat L, Goesmann A, Hartmann M, Huthmacher K, Krämer R, Linke B, McHardy AC, Meyer F, Möckel B, Pfefferle W, Pühler A, Rey DA, Rückert C, Rupp O, Sahm H, Wendisch VF, Wiegräbe I, Tauch A. The complete Corynebacterium glutamicum ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. J Biotechnol. 2003 4;104(1-3):5-25.

Kocijancic D, Felgner S, Frahm M, Komoll RM, Iljazovic A, Pawar V, et al. Therapy of solid tumors using probiotic Symbioflor-2: restraints and potential. Oncotarget. 2016;7:22605–22.

Kuhnert P, Nicolet J, Frey J. Rapid and accurate identification of Escherichia coli K-12 strains. Appl Environ Microbiol. 1995;61(11):4135-9.

Liu D. Reeves PR. *Escherichia coli* K12 regains its O antigen. Microbiol. 1994; 140:49–57.

National Institutes of Health. 2019. NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH Guidelines). Available at https://osp.od.nih.gov/wp-content/uploads/NIH_Guidelines.html, December 20, 2019.

Smith, H. W. 1975. Survival of orally administered E. coli K12 in alimentary tract of human. Nature (London) 255:500–502.

Steyn PL, Segers P, Vancanneyt M, Sandra P, Kersters K, Joubert JJ. Classification of heparinolytic bacteria into a new genus, *Pedobacter*, comprising four species: *Pedobacter heparinus* comb. nov., *Pedobacter piscium* comb. nov., *Pedobacter africanus* sp. nov. and *Pedobacter saltans* sp. nov. proposal of the family Sphingobacteriaceae fam. nov. Int J Syst Bacteriol. 1998;48 Pt 1:165-77.

Wassenaar TM, Zimmermann K. Lipopolysaccharides in Food, Food Supplements, and Probiotics: Should We be Worried? Eur J Microbiol Immunol (Bp). 2018;8(3):63-9.

Toxicology

24. *In the unpublished acute toxicity study (Appendix J), the notifier reports that the body weight gain was significantly suppressed in male rats at the highest dose, 7500 mg/kg.*

    However, the notifier concludes (page 48) that the LD50 is greater than 7500mg/kg. The notifier should clarify whether they consider the body weight effect in male rats is incidental and NOT test article-related and explain why. We note that the same effect was not observed in females of the same study, nor in males and females received the same dose following repeated-dose treatment, as seen in the subchronic study (Appendix J).

    Response

    We consider that a significantly suppressed body weight gain observed in male rats at the highest dose, 7500 mg/kg, in an acute toxicity study was incidental and not test article-related because the same effect was not observed in females of the same study, nor in males and females receiving the same dose following a repeated-dose treatment as seen in the subchronic toxicity study.

25. *The notifier's literature search covers publications through December 2019 and the notice was submitted in March 2020. The notifier should clarify if there are any publications between December 2019 and March 2020 that suggest any safety issues of 2'-FL, which may conflict with their GRAS conclusion.*

    Response

    We have identified two articles published between December 2019 to March 2020: a human observational study by Berger et al. (2020) and a series of toxicity studies of 2'-FL as part of a five HMO mixture by Parschat et al. (2020). The content of the 2020 study by Berger et al. was published in 2016 in an abstract form (Berger et al., 2016). Thus, this study is very briefly summarized in this response. The study by Parschat et al. (2020) evaluated the toxicity of the 5 HMO mixture, which is composed of 47.1% 2'-FL. Thus, this study is summarized below.

    A Human Clinical Study by Berger et al. (2020)

    Berger et al. (2020) examined whether HMOs influenced fecal microbiota by changing the fecal community types in term infants, and whether that would reduce antibiotic requirement associated with the risk of infections later. They performed a randomized, double-blind, multicenter clinical trial with 175 healthy full-term infants that were exclusively formula fed

since birth (37 – 42 weeks gestational age, 2.5 – 4.5 kg body weight, less than 2 weeks of age). These infants were randomized to receive either control (n=87) or test formulas (n=88) from enrollment until 6 months. The test infant formula contained two HMOs (1 g/L 2'-FL and 0.5 g/L LNnT). In addition, a reference group was comprised of infants who were exclusively breastfed since birth (n=38). Then both groups received infant formula without HMOs until 12 months. The breastfeeding group was exclusively breastfed until 4 months of age, and then complementary solid food was permitted. Supplementation of 2'-FL and LNnT had no adverse effects on the measured outcomes, such as fecal microbiota profiling on stool samples which were collected at 3 and 12 months of age. In addition, there was no adverse effects on antibiotic requirement associated with the risk of infections. The content of this paper was reported in 2016 in an abstract form (Berger et al., 2016).

Toxicological Evaluation of 2'-FL as Part of a Five HMO Mixture by Parschat et al. (2020)

Parschat et al. (2020) performed a series of toxicity studies of a mixture of 5 HMO (2'-FL, 3-FL, LNnT, 3'-SL, and 6'-SL). The HMO mixture contained 47.1% 2'-FL, 16.0% 3-FL, 23.7% LNnT, 4.1% 3'-SL, 4.0% 6'-SL, and 5.1% other carbohydrates on a dry weight basis.

Bacterial Reverse Mutation Test

To evaluate the potential of HMO mix to induce genetic mutation, the reverse mutation test in *Salmonella typhimurium* was performed in strains TA98, TA100, TA102, TA1535, and TA1537 in both a plate incorporation test and preincubation test, each performed in triplicate, with and without metabolic activation with S9 mix. Six concentrations of 5.0, 10.0, 31.6, 100, 316, or 600 mg HMO mix (equivalent to 2.4, 4.7, 14.9, 47.1, 148.8, or 282.6 mg 2'-FL)were applied to each plate. Purified water was used as a negative control. Appropriate positive controls were performed with and without metabolic activation. Cytotoxicity was defined as reduction of more than 50% colonies compared to solvent control. A 1.5- to 2-fold increase in the number of revertant compared to the solvent control (at least 2-fold for TA98, TA100, TA1535, and TA1537, and 1.5-fold for TA102) was considered mutagenic. Compared to the control, the positive controls had at least threefold increased mean revertant colony numbers, confirming the sensitivity of the assays. There were no signs of cytotoxicity or mutagenicity noted in any test strains up to 600 mg HMO mixture per plate (providing up to 282.6 mg 2'-FL per plate) in both the plate incorporation and preincubation tests. The authors concluded that the HMO mixture was not mutagenic under the test conditions.

Micronucleus Test in Cultured Human Peripheral Lymphocytes

An *in vitro* micronucleus test was performed using human peripheral blood lymphocytes (obtained from healthy, non-smoking individuals) in the presence and absence of metabolic activation with S9 mix. Each test was carried out in duplicate without S9 mix for 4 and 24 hours and with S9 mix for 4 hours only, all performed at 37°C at concentrations of 7.5, 15, 30, and 60 mg HMO mix/mL medium (equivalent to 3.5, 7.1, 14.1, and 28.3 mg 2'-FL/mL medium). Purified water was used as a negative control; mitomycin C and colchicine were used as positive controls without S9, and cyclophosphamide was the positive control in the presence of S9 mix. At least 500 cells per replicate cell culture were scored and estimated the proliferation index as a measure of toxicity after classifying them as mononucleates, binucleates or multinucleates. The evaluation of cytotoxicity was based on the Cytokinesis-Block Proliferation Index (CBPI) or the Replicative Index (RI). The micronucleus frequencies were analyzed in at least 2,000 binucleate cells per concentration (≥ 1000 binucleate cells per culture; two cultures per concentration). A test substance was considered to be positive if

there was a statistically significant and/or dose related increase compared to the negative control or if any of the results were outside the distribution of the historical negative control data (Poisson-based 95% control limits). There were no indications of chromosomal damage. The test was considered valid because the frequency of micronucleate cells for the test substance and the vehicle controls were within the historical control range, while positive controls, mitomycin C and cyclophosphamide induced significant chromosomal damage and colchicine induced damage to the cell division apparatus. The authors concluded that the HMO mixture was not genotoxic under the test conditions.

Subacute Toxicity Study in Rats

A 7-day pilot study was performed in 10 healthy female CD rats to determine the design of the subchronic repeated dose study (60 days old, 217.9-247.5 g body weight). The rats received one of two diets: standard diet (control) or diet supplemented with 10% HMO mix after 17.5 hour fasting. Feed and water were provided *ad libitum.* Rats were observed for clinical signs of toxicity, body weight, and feed and water intakes. There were no premature mortalities, and no changes in behavior, stool, body weight, body weight gain, or food consumption. Therefore, 10% HMO mix was considered for the 13-week study.

Subchronic Toxicity Study in Rats

A subchronic oral toxicity study was performed in 40 healthy CD rats (20 males and 20 females; 65 days old at the time of first treatment; males 343.1-390.5 g body weight; females 212.7-245.5 g body weight) for 13 weeks. The rats were divided into standard diet (control) and 10% HMO mix supplemented diet groups. The HMO mixture contained 47.1% dry weight 2'-FL; the overall dietary exposure to 2'-FL was 4.71% of diet. Feed and water were provided *ad libitum.* Rats were observed daily for clinical signs. Intake of food, water, and test material were recorded daily. Body weight and body weight gain were assessed weekly. In the 13th week, all animals were tested for sensory reactivity to different stimuli in order to assess neurological function. Eye and ear function were examined before test material administration and at 12 weeks. Blood and urine samples were taken from all animals at the end of the study and before necropsy on the final day. Organ weights and histopathology were assessed.

There were no treatment-related abnormalities in feed and water consumption, body weight, body weight gain, clinical signs, ophthalmological findings, clinical pathological parameters, stool consistency, body posture, movement, or coordination. All clinical parameters were in the normal range for all 13 weeks. There were no test-related effects in functional observation tests, grip strength tests, or spontaneous mobility tests.

There was a small but significant increase in body temperature in female test rats compared to the control (38.5 C vs. 38.1 C; $P<0.05$), but the change was approximately 1% and unassociated with other symptoms, and therefore, considered incidental and unrelated to treatment. For the neutrophils, the mean cell counts were generally low relative to the historical control range for the laboratory (0.4–12.81 × 10^3 cells/μL) in both the control and test groups. Additionally, female rats in both the control and test groups had generally low mean neutrophil counts relative to the historical control range for the laboratory (0.4–12.81 × 10^3 cells/μL), and the absolute number in one female in the test group fell below the lower boundary of the historical control range (0.33 × 10^3 cells/μL). However, all neutrophil counts in the remaining males and females fell within the historical range. Therefore, the statistically significant reduction in absolute number of neutrophilic granulocytes observed in the female

test group was not considered treatment-related. Likewise, there were no test material-related abnormalities in clinical chemistry parameters. Test males did have significant increases in fasting plasma HDL-C level, and test females had significant increases in plasma levels of albumin, globulin, protein, urea, and albumin/globulin ratio ($P<0.05$). However, they were deemed unrelated to the treatment because these values were within the historical range for the laboratory and the changes were less than 15%. There were no significant changes in urinalysis, organ weights, or histopathological changes.

In summary, the HMO mix did not induce genotoxicity, nor did it show adverse effects in a repeated dose study. Therefore, the NOAEL is considered to be 10% of the diet, which is equivalent to 5.67 g HMO mix/kg bw/day for males and 6.97 g HMO mix/kg bw/day for females. Corresponding 2'-FL levels were 2,671 and 3,283 mg 2'-FL/kg bw/day for male and female rats, respectively.

Reference

Berger PK, Plows JF, Jones RB, Alderete TL, Yonemitsu C, Poulsen M, Ryoo JH, Peterson BS, Bode L, Goran MI. Human milk oligosaccharide 2'-fucosyllactose links feedings at 1 month to cognitive development at 24 months in infants of normal and overweight mothers. PLoS One. 2020;15(2):e0228323.

Berger B, Sprenger N, Grathwohl D, Alliet P, Puccio G, Steenhout P, Lausanne NSA. Stool microbiota in term infants fed formula supplemented with synthetic human milk oligosaccharides is associated with reduced likelihood of medication. J Pediatric Gastroenterol Nutr. Abstract 1190. J Pediatr Gastroenterol Nutr. 2016;63 Suppl 2:S406

Parschat K, Oehme A, Leuschner J, Jennewein S, Parkot J. A safety evaluation of mixed human milk oligosaccharides in rats. Food Chem Toxicol. 2020;136:111118.

26. *On page 49, under the subtitle of "An Acute Toxicity Study of APTech's 2′-FL, continued", no discussion on the acute toxicity is provided but instead there is a discussion of the* 90-day toxicological study. Is there anything missing from the acute toxicity study?

Response

An acute toxicity study was described starting on page 48 of the original submission. Page 49 continued the discussion about suppression of body weight gain observed in an acute toxicity study by comparing with subchronic toxicity study to conclude that suppression of body weight gain observed in males in an acute toxicity study was incidental because the same effect was not found in females and in the subchronic toxicity study.

If the page 49 content were placed right after the following paragraph, it would have been less confusing.

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). -- 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).'

We would like to modify the paragraph on page 49 with modifications right after the above paragraph.

'As discussed for the 90-day oral toxicity study in rats (page 49), a transient body weight decrease was noted in males in the mid- and high-dose groups on days 11 and 4, respectively, and normalized starting day 15. These changes were not considered test substance-related because they were temporary, with little difference when compared to the control group. 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).'

We hope that the information above responds fully to FDA's follow-up question number 17 regarding GRAS Notification 932. We would be happy to provide you with any further information you may need.

Sincerely,

Susan S. Cho, Ph.D.
Susanscho1@yahoo.com
(301)875-6454

Appendix R.A. Certificates of Analysis

eurofins | Food Integrity & Innovation

Report Number:	**2821350-0**
Report Date:	**20-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-CG011**	**Eurofins Sample:**	**9351924**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	Result
ISO Enterobacteriaceae	
Enterobacteriaceae	Absent /10 g

Method References	Testing Location
ISO Enterobacteriaceae (EBISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

ISO 21528-1: Microbiology of food and animal feeding stuffs- Horizontal methods for the detection and enumeration of Enerobacteriaceae- Part 1: Detection and enumeration by MPN technique with pre-enrichment.

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:	**2821351-0**
Report Date:	**20-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-CG012**	**Eurofins Sample:**	**9351928**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	Result
ISO Enterobacteriaceae	
Enterobacteriaceae	Absent /10 g

Method References	Testing Location
ISO Enterobacteriaceae (EBISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

ISO 21528-1: Microbiology of food and animal feeding stuffs- Horizontal methods for the detection and enumeration of Enerobacteriaceae- Part 1: Detection and enumeration by MPN technique with pre-enrichment.

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:	**2821352-0**
Report Date:	**20-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-CG013**	**Eurofins Sample:**	**9351930**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	Result
ISO Enterobacteriaceae	
Enterobacteriaceae	Absent /10 g

Method References	Testing Location
ISO Enterobacteriaceae (EBISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

ISO 21528-1: Microbiology of food and animal feeding stuffs- Horizontal methods for the detection and enumeration of Enerobacteriaceae- Part 1: Detection and enumeration by MPN technique with pre-enrichment.

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:	**2821353-0**
Report Date:	**20-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:**	**9351932**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	Result
ISO Enterobacteriaceae	
Enterobacteriaceae	Absent /10 g

Method References	Testing Location
ISO Enterobacteriaceae (EBISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

ISO 21528-1: Microbiology of food and animal feeding stuffs- Horizontal methods for the detection and enumeration of Enerobacteriaceae- Part 1: Detection and enumeration by MPN technique with pre-enrichment.

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:	**2821354-0**
Report Date:	**20-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:**	**9351934**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	Result
ISO Enterobacteriaceae	
Enterobacteriaceae	Absent /10 g

Method References	Testing Location
ISO Enterobacteriaceae (EBISO)	**Food Integ. Innovation-Madison NE** 2102 Wright Street Madison, WI 53704 USA

ISO 21528-1: Microbiology of food and animal feeding stuffs- Horizontal methods for the detection and enumeration of Enerobacteriaceae- Part 1: Detection and enumeration by MPN technique with pre-enrichment.

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:	2827986-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825479-0

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:**	**9351924**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	LOQ	Limit	Result	Pass/Fail
Residual Solvents - Class 3				
1-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Pentanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
2-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
Methylethylketone	200 ppm	5000 ppm	<200 ppm	Pass
3-Methyl-1-butanol	200 ppm	5000 ppm	<200 ppm	Pass
Acetic Acid Butyl Ester	200 ppm	5000 ppm	<200 ppm	Pass
Acetone	200 ppm	5000 ppm	<200 ppm	Pass
Anisole	200 ppm	5000 ppm	<200 ppm	Pass
Diethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
Ethanol	200 ppm	5000 ppm	221 ppm	Pass
Ethyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Ethyl Formate	500 ppm	5000 ppm	<500 ppm	Pass
2-Methyl-1-propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isobutyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
2-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isopropyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methylisobutylketone	200 ppm	5000 ppm	<200 ppm	Pass
tert-Butylmethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
n-Heptane	200 ppm	5000 ppm	<200 ppm	Pass
n-Pentane	200 ppm	5000 ppm	<200 ppm	Pass
Propyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Total Class 3 Residual Solvents	5000 ppm	5000 ppm	<5000 ppm	Pass

Method References **Testing Location**

eurofins | Food Integrity & Innovation

Report Number:	2827986-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825479-0

Certificate of Analysis

Advanced Protein Technologies Corp.

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

Method References	Testing Location
Residual Solvents - Class 3 (USPR_S)	Food Integrity Innovation-Madison 3301 Kinsman Blvd Madison, WI 53704 USA

United States Pharmacopeia, 38nd Rev. - National Formulary 33th Ed., Method <467>, USP Convention, Inc., Rockville, MD (2015). (Modified).

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	Edward Ladwig - Director

Eurofins Food Chemistry Testing US, Inc.
3301 Kinsman Blvd
Madison WI 53704
800-675-8375

eurofins | Food Integrity & Innovation

Report Number:	2827987-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825480-0

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:**	**9351928**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	LOQ	Limit	Result	Pass/Fail
Residual Solvents - Class 3				
1-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Pentanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
2-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
Methylethylketone	200 ppm	5000 ppm	<200 ppm	Pass
3-Methyl-1-butanol	200 ppm	5000 ppm	<200 ppm	Pass
Acetic Acid Butyl Ester	200 ppm	5000 ppm	<200 ppm	Pass
Acetone	200 ppm	5000 ppm	<200 ppm	Pass
Anisole	200 ppm	5000 ppm	<200 ppm	Pass
Diethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
Ethanol	200 ppm	5000 ppm	220 ppm	Pass
Ethyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Ethyl Formate	500 ppm	5000 ppm	<500 ppm	Pass
2-Methyl-1-propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isobutyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
2-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isopropyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methylisobutylketone	200 ppm	5000 ppm	<200 ppm	Pass
tert-Butylmethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
n-Heptane	200 ppm	5000 ppm	<200 ppm	Pass
n-Pentane	200 ppm	5000 ppm	<200 ppm	Pass
Propyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Total Class 3 Residual Solvents	5000 ppm	5000 ppm	<5000 ppm	Pass

Method References **Testing Location**

eurofins | Food Integrity & Innovation

Report Number:	2827987-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825480-0

Certificate of Analysis

Advanced Protein Technologies Corp.

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

Method References	Testing Location
Residual Solvents - Class 3 (USPR_S)	**Food Integrity Innovation-Madison** 3301 Kinsman Blvd Madison, WI 53704 USA

United States Pharmacopeia, 38nd Rev. - National Formulary 33th Ed., Method <467>, USP Convention, Inc., Rockville, MD (2015). (Modified).

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	**Edward Ladwig - Director**

Eurofins Food Chemistry Testing US, Inc.
3301 Kinsman Blvd
Madison WI 53704
800-675-8375

eurofins | Food Integrity & Innovation

Report Number:	**2827988-0**
Report Date:	**27-Mar-2020**
Report Status:	**Final**
Supercedes :	**2825481-0**

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:**	**9351930**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	LOQ	Limit	Result	Pass/Fail
Residual Solvents - Class 3				
1-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Pentanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
2-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
Methylethylketone	200 ppm	5000 ppm	<200 ppm	Pass
3-Methyl-1-butanol	200 ppm	5000 ppm	<200 ppm	Pass
Acetic Acid Butyl Ester	200 ppm	5000 ppm	<200 ppm	Pass
Acetone	200 ppm	5000 ppm	<200 ppm	Pass
Anisole	200 ppm	5000 ppm	<200 ppm	Pass
Diethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
Ethanol	200 ppm	5000 ppm	221 ppm	Pass
Ethyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Ethyl Formate	500 ppm	5000 ppm	<500 ppm	Pass
2-Methyl-1-propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isobutyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
2-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isopropyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methylisobutylketone	200 ppm	5000 ppm	<200 ppm	Pass
tert-Butylmethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
n-Heptane	200 ppm	5000 ppm	<200 ppm	Pass
n-Pentane	200 ppm	5000 ppm	<200 ppm	Pass
Propyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Total Class 3 Residual Solvents	5000 ppm	5000 ppm	<5000 ppm	Pass

Method References **Testing Location**

Printed: 27-Mar-2020 9:45 am Page 1 of 2

eurofins | Food Integrity & Innovation

Report Number:	2827988-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825481-0

Certificate of Analysis

Advanced Protein Technologies Corp.

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

Method References	Testing Location
Residual Solvents - Class 3 (USPR_S)	**Food Integrity Innovation-Madison** 3301 Kinsman Blvd Madison, WI 53704 USA

United States Pharmacopeia, 38nd Rev. - National Formulary 33th Ed., Method <467>, USP Convention, Inc., Rockville, MD (2015). (Modified).

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	**Edward Ladwig - Director**

Eurofins Food Chemistry Testing US, Inc.
3301 Kinsman Blvd
Madison WI 53704
800-675-8375

eurofins | Food Integrity & Innovation

Report Number:	2827989-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825482-0

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:**	**9351932**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	LOQ	Limit	Result	Pass/Fail
Residual Solvents - Class 3				
1-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Pentanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
2-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
Methylethylketone	200 ppm	5000 ppm	<200 ppm	Pass
3-Methyl-1-butanol	200 ppm	5000 ppm	<200 ppm	Pass
Acetic Acid Butyl Ester	200 ppm	5000 ppm	<200 ppm	Pass
Acetone	200 ppm	5000 ppm	<200 ppm	Pass
Anisole	200 ppm	5000 ppm	<200 ppm	Pass
Diethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
Ethanol	200 ppm	5000 ppm	229 ppm	Pass
Ethyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Ethyl Formate	500 ppm	5000 ppm	<500 ppm	Pass
2-Methyl-1-propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isobutyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
2-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isopropyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methylisobutylketone	200 ppm	5000 ppm	<200 ppm	Pass
tert-Butylmethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
n-Heptane	200 ppm	5000 ppm	<200 ppm	Pass
n-Pentane	200 ppm	5000 ppm	<200 ppm	Pass
Propyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Total Class 3 Residual Solvents	5000 ppm	5000 ppm	<5000 ppm	Pass

Method References **Testing Location**

eurofins | Food Integrity & Innovation

Report Number:	2827989-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825482-0

Certificate of Analysis

Advanced Protein Technologies Corp.

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

Method References	Testing Location
Residual Solvents - Class 3 (USPR_S)	**Food Integrity Innovation-Madison** 3301 Kinsman Blvd Madison, WI 53704 USA

United States Pharmacopeia, 38nd Rev. - National Formulary 33th Ed., Method <467>, USP Convention, Inc., Rockville, MD (2015). (Modified).

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	**Edward Ladwig - Director**

Eurofins Food Chemistry Testing US, Inc.
3301 Kinsman Blvd
Madison WI 53704
800-675-8375

eurofins | Food Integrity & Innovation

Report Number:	2827990-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825483-0

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:**	**9351934**
Project ID	ADVAN_PR_T-20200310-0003	**Receipt Date**	16-Mar-2020
PO Number	APtech_20200311	**Receipt Condition**	Ambient temperature
Sample Serving Size	30 g	**Login Date**	10-Mar-2020
Description	Food grade(powder)	**Date Started**	16-Mar-2020
		Sampled	Sample results apply as received
		Online Order	15841-1322C659

Analysis	LOQ	Limit	Result	Pass/Fail
Residual Solvents - Class 3				
1-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Pentanol	200 ppm	5000 ppm	<200 ppm	Pass
1-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
2-Butanol	200 ppm	5000 ppm	<200 ppm	Pass
Methylethylketone	200 ppm	5000 ppm	<200 ppm	Pass
3-Methyl-1-butanol	200 ppm	5000 ppm	<200 ppm	Pass
Acetic Acid Butyl Ester	200 ppm	5000 ppm	<200 ppm	Pass
Acetone	200 ppm	5000 ppm	<200 ppm	Pass
Anisole	200 ppm	5000 ppm	<200 ppm	Pass
Diethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
Ethanol	200 ppm	5000 ppm	232 ppm	Pass
Ethyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Ethyl Formate	500 ppm	5000 ppm	<500 ppm	Pass
2-Methyl-1-propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isobutyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
2-Propanol	200 ppm	5000 ppm	<200 ppm	Pass
Isopropyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Methylisobutylketone	200 ppm	5000 ppm	<200 ppm	Pass
tert-Butylmethyl Ether	200 ppm	5000 ppm	<200 ppm	Pass
n-Heptane	200 ppm	5000 ppm	<200 ppm	Pass
n-Pentane	200 ppm	5000 ppm	<200 ppm	Pass
Propyl Acetate	200 ppm	5000 ppm	<200 ppm	Pass
Total Class 3 Residual Solvents	5000 ppm	5000 ppm	<5000 ppm	Pass

Method References **Testing Location**

eurofins | Food Integrity & Innovation

Report Number:	2827990-0
Report Date:	27-Mar-2020
Report Status:	Final
Supercedes :	2825483-0

Certificate of Analysis

Advanced Protein Technologies Corp.

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

Method References	Testing Location
Residual Solvents - Class 3 (USPR_S)	Food Integrity Innovation-Madison 3301 Kinsman Blvd Madison, WI 53704 USA

United States Pharmacopeia, 38nd Rev. - National Formulary 33th Ed., Method <467>, USP Convention, Inc., Rockville, MD (2015). (Modified).

Testing Location(s)	Released on Behalf of Eurofins by
Food Integrity Innovation-Madison	Edward Ladwig - Director

Eurofins Food Chemistry Testing US, Inc.
3301 Kinsman Blvd
Madison WI 53704
800-675-8375

Appendix R.B. Evaluation of horizontal gene transfer frequency of *Corynebacterium glutamicum* APC199 by conjugation with standard microorganisms

Final Report

Evaluation of horizontal gene transfer frequency of *Corynebacterium glutamicum* APC199 by conjugation with twenty microorganisms

Study organization: Bio-Evaluation Center,
Korea Institute of Bioscience and Biotechnology (KRIBB)

Sponsor: Advanced Protein Technologies Corp.

Table of Content

Declaration

Title of study: Evaluation of horizontal gene transfer rate of *Corynebacterium glutamicum* APC199 by conjugation with twenty microorganisms

This study was performed to evaluate the horizontal gene transfer capability of *C. glutamicum* APC199 to transfer antibiotic resistance genes to other bacteria because the strain of *C. glutamicum* APC199, a transformant of *C. glutamicum* ATCC13032 with the pFP110 plasmid, was developed for the fermentative production of 2'-fucosyllactose. As part of the overall safety assessment of bacteria used to produce food ingredients, one of the standard protocols is to determine if they are capable of allowing horizontal transfer of antibiotics genes. Whole genome sequencing of *C. glutamicum* APC199 and ATCC13032 strains did not find antibiotic resistance genes associated with the bacterium's chromosome. Antibiotic resistance gene was limited to the kanamycin resistance associated with the *nptII* gene on the pFP110 plasmid. This was used to estimate the conjugation-mediated horizontal gene transfer potential from *C. glutamicum* APC199 to other bacteria.

2018. 12. 19.

Ju Seok Lee, Ph.D.
Senior Researcher

Date

Address: 30, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongu-si, Chungcheongbuk-do, 28116, Republic of Korea

Contacts: (Tel) 82-43-240-6526, (Fax) 82-43-240-6549
E-mail: juseoklee@kribb.re.kr

Overview of study

Title of study: Evaluation of horizontal gene transfer frequency of APC199 by conjugation with twenty microorganisms

Objectives of study: For the evaluation of horizontal gene transfer frequency of APC199 by conjugation with twenty microorganisms

Sponsor: Advanced Protein Technologies Corp.
Address: 7th floor, 147, Gwanggyo-ro, Yeongtong-gu, Suwon-si, Gyeonggi-do, 16229, Republic of Korea

Study organization: Bio-Evaluation Center, Korea Institute of Bioscience and Biotechnology (KRIBB)
Address: 30, Yeongudanji-ro, Ochang-eup, Cheongwon-gu, Cheongu-si, Chungcheongbuk-do, 28116, Republic of Korea
Principal investigator: Ju Seok Lee, Ph.D.
Contacts: (Tel) 82-43-240-6526, (Fax) 82-43-240-6549

Study schedule
May 3, 2018 – Reception of study strains (APC199, *C. glutamicum* ATCC13032)
August 22, 2018 – Collection of standard microorganisms for study
October 30, 2018 – Completion of strain preparation of rifampicin-resistant strains
November 30, 2018 – Completion of conjugation tests by filter-mating method
December 15, 2028 – Submission of final report to sponsor

Research scientists

Ju-Hyun Im – LMO Risk Assessment, Conjugation experiments
Ju Seok Lee – LMO Risk Assessment, Experiment design and analysis

1. Summary

For the evaluation of gene transfer capability of plasmid DNA pFP110 of genetically modified *Corynebacterium glutamicum* APC199 strain by conjugation with microorganisms in the environment, we examined plasmid DNA reception by twenty microorganisms (commonly used, related, commonly found from soil, water, and/or human, and potential pathogenic microorganisms, Table 1) using filter-mating method. To discriminate donor microorganism (APC199), which has kanamycin resistance associated with the *nptII* gene, from recipient microorganisms (twenty tested microorganisms), the *rpoB* gene of recipient microorganisms was mutated by the treatment of mutagen ethyl methanesulfonate (EMS) to give resistance to rifampicin. Gene transfer frequencies by conjugation were evaluated by the calculation of colony counting of recipient microorganisms showing dual antibiotic resistance (kanamycin and rifampicin). The gene transfer frequency values by conjugation among the twenty tested microorganisms were below or close to the detection limit of 1.1×10^{-10}; no dual antibiotic resistant microorganisms were observed among the twenty tested strains (Table 3). These frequencies are about 10 times lower than the minimum transfer frequencies of 1.3×10^{-9} derived from literature search (Table 2). The frequencies of development of dual antibiotic resistance observed are more consistent with the development of spontaneous mutation leading to antibiotic resistance that is often associated with a variety of bacteria. There would be substantially higher frequencies if target horizontal gene transfer was occurring via conjugation. It is concluded that there is a very low likelihood of plasmid transfer because the transfer frequencies found in this study were consistent with spontaneous mutations instead of conjugal plasmid transfer.

2. Objectives of study

Genetically modified microorganism APC199 shows kanamycin resistance because the stain harbors pFP110 plasmid DNA, which contains *nptII* gene as a selection marker. This plasmid DNA can be transferred to other microorganisms in contact by naturally occurring conjugation. We have evaluated the horizontal gene transfer frequencies of APC199 by calculating the occurrence of colonies showing dual antibiotic resistance for rifampicin and kanamycin by filter-mating conjugation between APC199 and recipient microorganism having rifampicin resistance.

3. Materials and Methods

3.1. Twenty microorganisms

We have tested twenty microorganisms (commonly used, related, commonly found in soil or water, and potentially pathogenic) for the evaluation of gene transfer frequency of recombinant plasmid DNA pFP110 to recipient microorganisms (Table 1). The twenty test strains were consisted of typical microorganisms from various sources such as soil, water, human and pathogen. Corynebacterium strains were included in the test as related microorganisms with APC 199.

Table 1. List of twenty microorganisms for the evaluation of horizontal gene transfer

Type	Recipient microorganism	Strain No.	Culture condition [a)]	
			Media	Temp. (°C)
Commonly used	*Bacillus subtilis*	KCTC 2189	Nutrient	30
	Escherichia coli K12	KRIBB stock	Luria-Bertani	37
Related	*Corynebacterium acetoacidophilum*	KCCM 11411	Brain Heart Infusion	30
	Corynebacterium ammoniagenes	KCCM 11740	Nutrient	30
	Corynebacterium doosanense	KCTC 19568	Tryptic Soy	30
	Corynebacterium glutamicum	KRIBB stock	Nutrient	30
	Corynebacterium mycetoides	KCCM 12458	Nutrient	30
	Corynebacterium xerosis	KCCM 40941	Brain Heart Infusion	37
Commonly found in soil or water	*Acinetobacter sp.*	KCTC 12681	Nutrient	26
	Bacillus megaterium	KCTC 3712	Nutrient	30
	Paenibacillus polymyxa	KCTC 3717	Nutrient	37
	Pseudomonas putida	KCTC 2940	Luria-Bertani	26
	Flavobacterium sp.	KCTC 22205	Marine	25
	Proteus mirabilis	KCTC 2510	Nutrient	37
Potentially pathogenic	*Micrococcus luteus*	KRIBB stock	Enriched Nutrient	30
	Bacillus cereus	KRIBB stock	Nutrient	30
	Pasteurella pneumotropica	KRIBB stock	Brain Heart Infusion	37
	Shigella sonnei (Levine) Weldin	KCTC 22530	Nutrient	37
	Pseudomonas aeruginosa	KRIBB stock	Nutrient	37
	Staphylococcus aureus	KRIBB stock	Luria-Bertani	37

[a)] Shaking incubation at 130 rpm, 24 hours.

3.2. Preparation of rifampicin resistant strains by ethyl methanesulfonate (EMS)

For the preparation of rifampicin resistant strains of the twenty test microorganisms, the strains were cultured in liquid media for 12 hours, and 1% (v/v) of each culture was transferred to new liquid media and cultured for additional 4 hours. Grown cells were spun down by centrifugation for 10 minutes at 3,000 rpm and washed twice with Dulbecco's Phosphate Buffered Saline (DPBS). After incubation for 1 hour in DPBS containing 0.2% (v/v) EMS at temperatures specific for each recipient microorganism, EMS was removed by washing with DPBS. Rifampicin resistant colonies were selected by cultivation of each strain in specific growth media containing 100 mg/ml rifampicin for 12 hours. Single colony of each strain was grown in liquid media containing 100 mg/ml rifampicin for 12 hours, and harvested cells were mixed with 25% glycerol for frozen cell stock and kept in -80°C freezer. These rifampicin resistant strains of recipient microorganisms were used for the filter-mating conjugation tests. Rifampicin resistance of each strain was confirmed by growth on agar plate containing rifampicin. An example of *Proteus mirabilis* is shown in Figure 1.

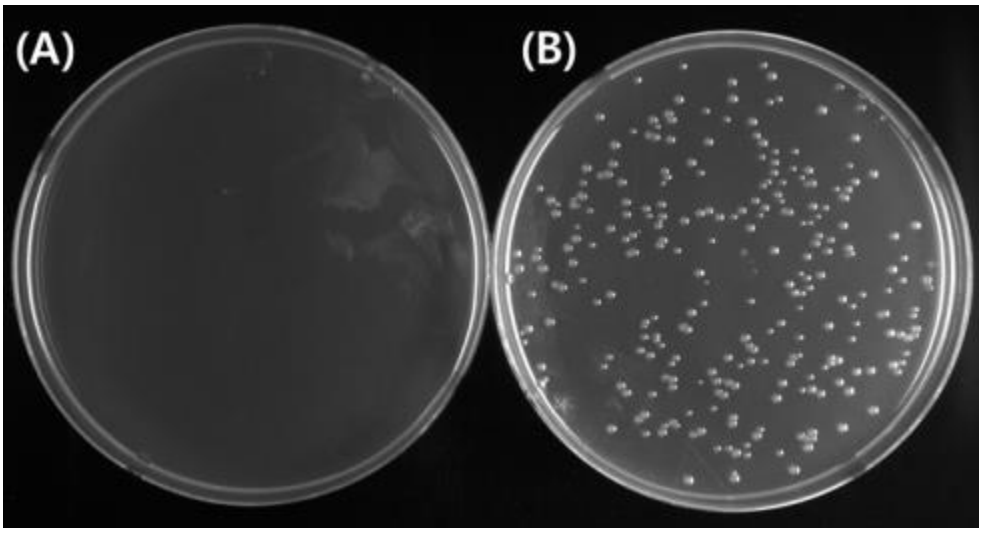

Figure 1. Growth of wild type *Proteus mirabilis* (A) and rifampicin resistant *Proteus mirabilis* obtained by EMS treatment (B) on agar plate containing 100 mg/ml rifampicin.

3.3. Conjugation tests by filter-mating method

Filter-mating method was used for the evaluation of horizontal gene transfer of APC199 to recipient microorganisms. This method is considered more efficient than liquid-mating or solid-mating method for the evaluation of horizontal gene transfer because plasmid DNA transfer by conjugation occurs by physical contact between donor-recipient bacterial cells (Lampkowska et al., 2008). Higher chance of density and duration of physical contact in filter-mating than random contact in liquid culture explains the better efficiency of filter-mating method for gene transfer test. For the conjugation tests, donor and recipient microorganisms were cultured in 100 ml for 12 hours at each optimal temperature. Colonies were counted for the serial diluents of recipient microorganisms at 10^{-6} - 10^{-12} and grown on agar plate containing rifampicin. Cultured cells were harvested and washed twice with phosphate-buffered saline (PBS) for the removal of kanamycin and rifampicin. PBS washed APC199 cells were mixed with recipient cells and divided in three 50 ml tubes. The mixtures in each tube were washed again with PBS, and the final volume was adjusted to 300 ml with PBS. Cell mixtures of APC199 and each recipient microorganisms were placed on filter membranes (25 mm, pore size 0.2 mm, ADVANTEC) and cultured on nutrient agar plate containing no antibiotics for 24 hours at optimal temperatures. The filter membrane was transferred to 50 ml tube, and conjugated cells were separated from the filter membrane. To check the gene transfer of pFP110 DNA of APC199 to recipient microorganisms, the conjugated cells were grown on each specific agar plate containing both rifampicin and kanamycin. If the antibiotic resistance (kanamycin resistance) of APC199 was transferred to the recipient strain by conjugation, the resulting strain will acquire dual antibiotic resistance and will grow on agar plate containing kanamycin and rifampicin. The frequencies of horizontal gene transfer were calculated by counting colonies showing dual antibiotic resistance (rifampicin and kanamycin) divided by initial number of cells of recipient microorganisms added to the conjugation tests.

4. Results

4.1. Plasmid DNA transfer frequency by filter-mating method

It is considered that there is no internationally established guideline for safe range of gene transfer frequency. We have searched for literatures regarding the study of gene transfer frequencies and found no case of gene transfer frequencies lower than 1.3×10^{-9}. We used this information for the evaluation of this study (Table 2). The results of plasmid DNA transfer

frequencies of APC199 to twenty recipient microorganisms by filter-mating methods are summarized in Table 3. The gene transfer frequencies by conjugation among the twenty tested microorganisms were below or close to the detection limit of 1.1×10^{-10} (Table 3). These frequencies are about 10 times lower than the minimum transfer frequencies of 1.3×10^{-9} derived from literature search (Table 2).

The frequencies of dual antibiotic resistance observed are more consistent with the development of spontaneous mutation (Nyinoh, 2019; O'Neill et al., 2001). There would be substantially higher frequencies if target horizontal gene transfer occurred via conjugation. Thus, it is concluded that there is a very low likelihood of plasmid transfer because the transfer frequencies found in this study were consistent with spontaneous mutations instead of conjugal plasmid transfer.

Table 2. Plasmid DNA transfer frequencies between donor and recipient microorganisms in environments (from literature search)

Donor microorganism	Recipient microorganism	Plasmid	Conjugation method	Gene transfer frequencies	Ref.
Lactobacillus lactis SH4147	*Lactobacillus lactis* subsp. *lactis*	pAMb1	Filter-mating	6.0×10^{-3}	Lampkowska et al., 2008
			Solid-mating	1.4×10^{-3}	
			Liquid-mating	2.3×10^{-7}	
Tetracycline-resistant *Lactobacillus*	*Enterococcus faecalis* JH2-2	natural	Filter-mating	6.5×10^{-5}	Gevers et al., 2003
	Lactose-negative *Lactobacillus lactis* subsp. *lactis* BU2-60	natural	Filter-mating	1.3×10^{-5}	
Pseudomonas putida Cg1	*Pseudomonas putida* KT2440-Tc	pCg1	Solid-mating	2.7×10^{-7}	Park et al., 2003
			Liquid-mating	1.3×10^{-9}	
Streptococcus faecalis	*Lactobacillus plantarum*	pAMb1	Filter-mating	7.4×10^{-8}	Sasaki et al., 1988

Table 3. Gene transfer frequencies of pFP110 of *C. glutamicum* APC199 to twenty microorganisms

Type	Recipient microorganism	Test condition		Gene transfer frequencies [1)]
		Media	Temp. (°C)	
Commonly used	*Bacillus subtilis*	NB	30	$<1.4 \times 10^{-10}$
	Escherichia coli K12	LB	37	$<1.3 \times 10^{-10}$
Related	*Corynebacterium acetoacidophilum*	BHI	30	$<1.5 \times 10^{-10}$
	Corynebacterium ammoniagenes	NB	30	$<1.4 \times 10^{-10}$
	Corynebacterium doosanense	TSA	30	$<1.1 \times 10^{-10}$
	Corynebacterium glutamicum	NB	30	$<1.3 \times 10^{-10}$
	Corynebacterium mycetoides	NB	30	$<1.1 \times 10^{-10}$
	Corynebacterium xerosis	BHI	37	$<1.3 \times 10^{-10}$
Commonly found in soil or water	*Acinetobacter sp.*	NB	26	$<1.3 \times 10^{-10}$
	Bacillus megaterium	NB	30	$<1.2 \times 10^{-10}$
	Paenibacillus polymyxa	NB	37	$<1.2 \times 10^{-10}$
	Pseudomonas putida	LB	26	$<1.3 \times 10^{-10}$
	Flavobacterium sp.	Marine	25	$<1.4 \times 10^{-10}$

	Proteus mirabilis	NB	37	$<1.4 \times 10^{-10}$
Potentially pathogenic	*Micrococcus luteus*	ENB	30	$<1.4 \times 10^{-10}$
	Bacillus cereus	NB	30	$<1.4 \times 10^{-10}$
	Pasteurella pneumotropica	BHI	37	$<1.5 \times 10^{-10}$
	Shigella sonnei (Levine) Weldin	NB	37	$<1.2 \times 10^{-10}$
	Pseudomonas aeruginosa	NB	37	$<1.1 \times 10^{-10}$
	Staphylococcus aureus	LB	37	$<1.3 \times 10^{-10}$

[1)] Test results of triplicates.

5. References

1. Lampkowska J, Feld L, Monaghan,A, et al. A standardized conjugation protocol to asses antibiotic resistance transfer between lactococcal species. Int J Food Microbiol. 2008;127:172-5.
2. Gevers D, Huys G, Swings J. In vitro conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. FEMS Microbiol Lett. 2003;225:125-30.
3. Park W, Jeon CO, Hohnstock-Ashe AM, Winans S. C, Zylstra GJ, Madsen E L. Identification and Characterization of the Conjugal Transfer Region of the pCg1 plasmid from Naphthalene-Degrading *Pseudomonas putida* Cg1. Applied and Environmental Microbiol. 2003;69:3263-71.
4. Sasaki Y, Taketomo N, Sasaki T. Factors affecting transfer frequency of pAM beta 1 from *Streptococcus faecalis* to *Lactobacillus plantarum*. Journal of Bacteriol. 1988;170:5939-42.
5. Nyinoh IW. Spontaneous mutations conferring antibiotic resistance to antitubercular drugs at a range of concentrations in Mycobacterium smegmatis. Drug Dev Res. 2019;80(1):147-154.
6. O'Neill AJ, Cove JH, Chopra I. Mutation frequencies for resistance to fusidic acid and rifampicin in Staphylococcus aureus. J Antimicrob Chemother. 2001;47(5):647-50.

From:	Susan S Cho
To:	Hice, Stephanie
Subject:	Re: GRN 000932 - Response to FDA"s Questions
Date:	Thursday, December 3, 2020 3:45:57 PM
Attachments:	Response to FDA 2nd Questions -submitted to FDA 12-3-2020.docx image001.png

---

Dear Dr. Hice,

Please find APTech's response to FDA's questions in the attached document. We would appreciate your kind attention to this matter. Thank you

Sincerely,
Susan
Susan Cho, Ph.D.
NutraSource, Inc. (New company name, AceOne RS)
+1-410-531-3336 (O) +1-301-875-6454 (C)

On Monday, November 30, 2020, 05:37:19 PM EST, Hice, Stephanie <stephanie.hice@fda.hhs.gov> wrote:

Dear Dr. Cho,

During our review of GRAS Notice No. 000932, we noted further questions that need to be addressed and are attached to this email.

We respectfully request a response within **10 business days**. If you are unable to complete the response within that time frame, please contact me to discuss further options. Please do not include any confidential information in your response.

If you have questions or need further clarification, please feel free to contact me. Thank you in advance for your attention to our comments.

Sincerely,

Stephanie Hice

Stephanie Hice, PhD

Staff Fellow (Biologist)

Division of Food Ingredients

Center for Food Safety and Applied Nutrition

Office of Food Additive Safety

U.S. Food and Drug Administration

stephanie.hice@fda.hhs.gov

December 3, 2020

Dr. Stephanie Hice
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: Response to FDA's Additional Questions related to GRN 000932, 2'-fucosyllactose

From: Susan Cho, NutraSource, Inc. (new company name, AceOne RS)

Dear Dr. Hice,

In response to FDA's questions, we have prepared our response as follows.

FDA's Comment In points/questions 8 and 9
Comment In points/questions 8 and 9, we had requested that the notifier consider specifications that more closely reflected their results of batch analyses for protein and arsenic. The notifier declined based on specifications from other GRAS notices that received "no questions" letters. We remind the notifier that, as newer, more sensitive methods become available (e.g., as observed for contaminants in fish oils), accepted industry standards may change. While we do not question the safety of the notifier's ingredient based on the batch analyses provided, we do suggest that the notifier's specifications are based on their method of manufacture and industry standards rather than static information provided in previous GRAS notices. Further, as stated in our 2017 guidance,1 we remind the notifier that "… all GRAS conclusions must be considered in context based on the knowledge and information available at a point in time, because scientific knowledge and information about a particular substance can evolve and sometimes change over time."

APTech's Response
We concur with FDA's comments. APTech wants to lower the specifications for protein content, As, and other components present in 2'-FL as follows:
Protein content, ≤ 50 μg/g
As, ≤ 0.03 mg/kg
Lactose, ≤ 1%
Difucosyllactose (DFL), ≤ 2%

In addition, APTech wants to remove the specifications for 3-fucosyllactose (3-FL), fucosyl-galactose (FG), and fucose because there are no detectable levels of such carbohydrates in APTech's 2'-FL preparations. Regardless, the safety aspects of these carbohydrates are discussed in our response to FDA's question 3. We have updated the specifications, and the following table is the revised specifications of APTech's 2'-FL.

Revised 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	≤ 50	Bradford assay; Bio-rad protein assay #5000006
Total ash, %	≤ 0.5	AOAC 923.03 or equivalent
Arsenic, mg/kg	≤ 0.03	ISO 17294:2014 (modified)* or equivalent
Cadmium, mg/kg	≤ 0.01	
Lead, mg/kg	≤ 0.02	
Mercury, mg/kg	≤ 0.05	
2'-Fucosyllactose, %	≥ 94	Validated HPAEC-PAD
Lactose, %	≤ 1 (Area)	
Difucosyllactose, %	≤ 2 (Area)	
Glucose, %	≤ 3 (Area)	
Galactose, %	≤ 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
Enterobacteriaceae	Absent in 10 g	ISO 21528-1
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)
Residual ethanol, mg/kg	≤ 1,000	USP, 38nd Rev. – National Formulary 33th Ed., USP <467>, (2015). (modified).

Abbreviations: AOAC = Association of Official Analytical Chemists; ASTM = The American Society for Testing and Materials; cfu = colony forming units; EU=endotoxin unit; HPAEC-PAD = high performance anion exchange chromatography pulsed amperometric detection; IDF = International Dairy Federation; ISO = International Organization for Standardization; Ph. Eur = European Pharmacopoeia; USP = The United States Pharmacopeia.
*Sample preparation methods have been modified.

Questions 1. *Please confirm that the batch analyses presented in Table 6 (page 6 of the October 23, 2020 amendment) correspond to results for non-consecutive batches*

APTech's Response
Yes, the batch analyses presented in Table 6 correspond to the results for non-consecutive batches. Manufacturing dates for Batch No. 2'-FL-CG-011 to 015 were October 29, 2018, December 10, 2018, December 17, 2018, December 20, 2018, and January 2, 2019, respectively.

Question 2
Regarding question 5, the notifier cited GRN 000735 as a notice with comparable use levels for 2'-FL. We note that the cumulative uses in GRNs 000735 and 000932 do not include uses in certain food categories (e.g., carbonated beverages, flavored waters, and vegetable juices) included in other GRNs for 2'-FL. Please provide a brief statement that these uses not included in the notifier's estimate do not meaningfully affect overall estimates of exposure to 2'-FL in the total diet and do not affect their safety conclusions.

APTech's Response
The EDIs from intended use of APTech's 2'-FL and those from cumulative uses were compared using the 2017-2018 National Health and Nutrition Examination Survey (NHANES) dataset. The cumulative uses included those defined in other GRNs for 2′-FL, such as GRNs 000650, 000815, and 000897, in additional to intended uses described in GRNs 000735 and 000932. These additional intended uses and use levels are described at the end of Table 2.1.

Prior to this comparison, EDIs of APTech's intended use and use levels based on the 2013-2014 NHANES and 2017-2018 NHANES were compared to find out if confounding factors, such as differences in survey subjects, possible changes in Americans' dietary intake pattern over time, and analysts employed in the two dataset analysis, have significant impact on the EDI values. The EDIs based on the 2013-2014 NHANES dataset were adopted from GRN 000735, and the EDIs derived from the 2017-2018 dataset were calculated by AceOne RS. As shown in Tables 2.2. and 2.3., the EDIs derived from the two NHANES datasets were comparable. For example, the 90th percentile EDIs based on the 2017-2018 and 2013-2014 NHANES dataset were 3.32 and 3.54 g/person/day in all-users in all ages, which may correspond to 71 and 80 mg/kg bw/day, respectively.

After inclusion of 4 additional food categories, the cumulative EDIs were not significantly increased to meaningfully affect the overall estimates of exposure to 2′-FL in the total diet (Tables 2.4. and 2.5). For example, the 90th percentile EDIs of 2'-FL in all users in all ages were 3.32 and 3.59 g/person/day under the APTech's and cumulative intended uses, respectively. These levels may correspond to 71 and 74 mg/kg bw/day, respectively.

Thus, the cumulative EDI values do not affect the safety conclusions included in the original submission.

Table 2.1. 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)
GRNs 000932 and 000735				
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	0.28	240	0.12

	combinations, yogurt drinks, and fermented milk drinks including kefir			
	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
Additional Intended Use for Cumulative Exposure Calculations				
Beverage	Fruit based ades; soft drinks** (GRN 000815)	0.54	360 mL	1.5 g/L or 1.5 g/kg

	Meal replacement drinks for weight reduction (GRN 000650)	1.20	240 mL	5.0 g/L
Processed vegetables and vegetable juices	100% Vegetable juices (GRN 000897)			1.2 g/kg
Tube-feeding formulas	Nutritional beverages such as Boost, Ensure, and Glucema as surrogates for tube feeding formulas (GRN 000897)			20 g/kg

Adopted from GRN 000735 (pages 30 to 31), GRN 000815 (page 6), GRN 000897 (page 13), and GRN 000650 (page 2 or stamped page 8).
*Ready-to-drink or reconstituted formulas prepared from powder; NA=not applicable;
** The 2'-FL content of 2'-FL/difucosyllactose mixture was 75% or higher.

Table 2.2. EDIs Under the APTech's Intended Use in All Users, g/day

		Mean EDIs		90th percentile EDIs	
Population Group	Age Group	2017-18 NHANES	2013-14 NHANES	2017-18 NHANES	2013-14 NHANES
Infants	0-5 months	2.06	1.91	3.17	3.00
Infants	6-11 months	2.60	2.28	4.95	3.86
Toddlers	12-35 months	1.42	1.83	2.21	2.97
Children	3-11 years	1.67	1.97	3.11	3.53
Female Teenagers	12-19 years	1.76	1.55	3.72	2.95
Male Teenagers	12-19 years	1.69	2.00	3.32	4.29
Women of Child-Bearing Age	16-45 years	1.20	1.36	2.61	2.87
Female Adults	≥20 years	1.30	1.44	2.92	3.05
Male Adults	≥20 years	1.69	1.84	3.76	3.97
Elderly	≥65 years	1.70	1.90	4.09	3.91
Total Population	All ages	1.54	1.70	3.32	3.54

Table 2.3. EDIs Under the APTech's Intended Use in All Users, mg/kg bw Users only

		Mean EDIs		90th percentile EDIs	
Population Group	Age Group	2017-18 NHANES	2013-14 NHANES	2017-18 NHANES	2013-14 NHANES
Infants	0-5 months	329	315	529	532
Infants	6-11 months	290	259	532	447
Toddlers	12-35 months	117	148	187	243
Children	3-11 years	64	76	126	147
Female Teenagers	12-19 years	29	26	68	52
Male Teenagers	12-19 years	27	31	54	67
Women of Child-Bearing Age	16-45 years	17	20	37	43
Female Adults	≥20 years	18	20	42	43
Male Adults	≥20 years	20	22	47	48
Elderly	≥65 years	22	26	52	54
Total Population	All ages	31	36	71	80

Table 2.4. EDIs Under the APTech's and Cumulative Intended Uses in All Users, g/day

		Mean EDIs		90th percentile EDIs	
Population Group	Age Group	APTech	Cumulative	APTech	Cumulative
Infants	0-5 months	2.06	2.06	3.17	3.17
Infants	6-11 months	2.60	2.60	4.95	4.95
Toddlers	12-35 months	1.42	1.45	2.21	2.23
Children	3-11 years	1.67	1.77	3.11	3.33
Female Teenagers	12-19 years	1.76	1.91	3.72	3.79
Male Teenagers	12-19 years	1.69	1.95	3.32	3.71
Women of Child-Bearing Age	16-45 years	1.20	1.42	2.61	2.96
Female Adults	≥20 years	1.30	1.53	2.92	3.31
Male Adults	≥20 years	1.69	1.98	3.76	4.25
Elderly	≥65 years	1.70	1.96	4.09	4.49
Total Population	All ages	1.54	1.77	3.32	3.59

Based on the 2017-2018 NHANES dataset.

Table 2.5. EDIs Under the APTech's and Cumulative Intended Uses in All Users, mg/kg bw

		Mean EDIs		90th percentile EDIs	
Population Group	Age Group	APTech	Cumulative	APTech	Cumulative
Infants	0-5 months	329	329	529	529
Infants	6-11 months	290	290	532	532
Toddlers	12-35 months	117	119	187	187
Children	3-11 years	64	67	126	132
Female Teenagers	12-19 years	29	32	68	71
Male Teenagers	12-19 years	27	31	54	64
Women of Child-Bearing Age	16-45 years	17	20	37	43
Female Adults	≥20 years	18	21	42	45
Male Adults	≥20 years	20	23	47	51
Elderly	≥65 years	22	25	52	60
Total Population	All ages	31	34	71	74

Based on the 2017-2018 NHANES dataset.

References

FDA 2016. GRN 000650. 2'-*O*-fucosyllactose. Available online: https://wayback.archive-it.org/7993/20180123210742/https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/UCM513832.pdf.

FDA 2018. GRN 000735, 2'-fucosyllactose. Available online: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=735&sort=GRN_No&order=DESC&startrow=1&type=basic&search=735.

FDA, 2019. GRN 000815, 2'-fucosyllactose and difucosyllactose. Available online: https://www.cfsanappsexternal.fda.gov/scripts/ fdcc/index.cfm?set=GRASNotices&id=815.

FDA. 2020. GRN 000897. 2-O-fucosyllactose. Available online: https://www.fda.gov/media/136755/download.

Question 3
Regarding question 11, we request that the notifier provide a brief summary of the safety of the other components (3-FL, FG, and fucose) present to complete the information provided on page 25 of GRN 000932 regarding conclusions of the safety of minor components.

APTech's response
No 3-FL, FG, and fucose were detected from APTech's 2'-FL preparations. Thus, APTech believes that specifications for those carbohydrates are not necessary. Thus, those carbohydrates are not included in APTech's new specifications for 2'-FL (please see Revised Table 5 of this response document). Even if APTech's 2'-FL would contain close to 1% of each of those carbohydrates, it is expected that the presence of such carbohydrates would not adversely impact the safety of APTech's 2'-FL preparation for the following reasons:

3-FL
Natural presence in human milk: 3-FL is a naturally occurring oligosaccharide in human milk (Thurl et al., 2017). It is also found in the milk of cows (Aldredge et al., 2013). Thus, humans have been exposed to 3-FL either through the ingestion of milk from humans and/or cows.

Human milk is the reference standard for infant food or formula. Based on the pooled data from 10 studies reported in Thurl et al. (2017) and the 17 additional studies reported in GRN 000925, Jennewein summarized that the concentration of 3-FL in breast milk ranged from 0 to 5.9 g/L, with means and medians ranging from 0 to 2.4 g/L and 0 to 1.1 g/L, respectively, although 3-FL levels in breast milk vary with time postpartum, secretor status, geographical location, and study population (GRN 000925, page 15 to 18). For example, mean concentrations of 3-FL in breast milk samples from women in Asia and in the US were 1.38 g/L and 1.03 g/L, respectively, during the first 3-10 days and 2.15 g/L and 2.57 g/L, respectively, after the first month postpartum.

Even if APTech's 2'-FL would contain close to 1% 3-FL, the intended use of APTech's 2'-FL would result in the maximum concentration of 0.024 g/L in ready-to-drink or reconstituted formula, which is well within the levels human infants have been exposed.

Safety Studies
Mutagenicity, genotoxicity, and animal toxicity studies by several investigators support the safety of 3-FL. These studies are described in detail in GRN 000925. Therefore, this response incorporates, by reference, the safety and metabolic studies discussed in GRN 000925, and will not discuss previously reviewed references in detail. Briefly, the following studies were summarized in GRN 000925.

1) The study by Pitt et al. (2019) tested the safety of 3-FL preparation (containing 94.6% 3-FL and 1.2% fucose). No adverse effects of 3-FL were observed in the mutagenicity and genotoxicity tests (bacterial reverse mutation assay, mammalian cell micronucleus test in Chinese Hamster Ovary cells, chromosomal aberration test in human lymphocytes, and *in vivo* mouse micronucleus test) as well as acute toxicity (by gastric intubation) and dietary 90-day subchronic toxicity studies in rats. The data support the safety of 3-FL and a minor component, fucose. Subchronic exposure of rats to 3-FL at the levels of 5 and 10%

of the diet did not produce any treatment-related abnormalities in growth, food intake or efficiency, clinical observations, hematology, clinical chemistry, organ weights, or histopathology parameters at average daily intakes of 5,980 and 7,270 mg/kg bw/day for males and females, respectively. Thus, the NOAELs were placed at 5,980 and 7,270 mg/kg bw/day for male and female rats, respectively.

2) In addition, the diet containing 10% of an HMO mixture [47.1% 2'-fucosyllactose (2'-FL), 16.0% 3-FL, 23.7% lacto-N-tetraose (LNT), 4.1% 3'-sialyllactose (3'-SL), 4.0% 6'-sialyllactose (6'-SL), and 5.1% other carbohydrates on a dry weight basis], providing the overall dietary exposure to 3-FL as 1.6% of the diet. This HMO containing diet did not result in any adverse effects in mutagenicity and genotoxicity tests (reverse mutation test in *Salmonella typhimurium* and micronucleus test in cultured human peripheral lymphocytes) as well as in 7-day oral tolerance and 13-week dietary toxicity studies in rats (GRN 000925, pages 33 to 39; Parschat et al., 2020). Overall, no signs of toxicity were observed following the administration of a mixture of five HMOs, containing 2'-FL, 3-FL (16.0% 3-FL by dry weight), LNT, 3'-SL, and 6'-SL, at 10% of diet for 13 weeks. Mean intakes of the HMO mixture were 5,010 to 6,880 mg/kg bw/day in males and 6,260 to 7,910 mg/kg bw/day in females. Mean intakes of 3-FL ranged from 800 to 1,100 mg/kg bw/day in males and from 1,000 to 1,270 mg/kg bw/day in females. The NOAELs for the HMO mixture were placed at 5,670 and 6,970 mg/kg bw/day for the male and female rats, respectively. The NOAELs for 3-FL were 910 mg/kg bw/day in male rats and 1,120 mg/kg bw/day in female rats.

3) An unpublished neonatal piglet study (GRN 000925, pages 40 to 70) showed that daily dietary administration of an oligosaccharide blend (containing 49.1 % 2'-FL, 10.4 % 3-FL, 19.9% LNT, 3.5 % 3'-SL, and 4.17 % 6'-SL) in specialty milk replacer formula for 3 weeks following birth was well tolerated at concentrations of 5.75 or 8.0 g/L with no adverse effects on their growth and development.

Overall, the safety of 3-FL is supported by the history of safe use in humans via consumption of human milk and the safety studies described above.

APTech's 2'-FL does not contain 3-FL. Thus, 3-FL is not included in APTech's new specifications for 2'-FL. Even if it would contain less than 1% of 3-FL, the estimated mean and 90th percentile EDIs would be within the safe intake levels due to the following reasons: the intended use would result in fucose concentration at 0.024 g/L in ready-to-drink or reconstituted formula. This would result in the estimated mean and 90th percentile intakes of 2.6 and 4.3 mg/kg bw/day, respectively (we multiplied 0.01 to the estimated daily intake values of 2'-FL in infant formulas only in all-user infants aged 0 to 11.9 months). In infants, the estimated mean and 90th percentile intakes of 2'-FL were estimated to be 258.7 and 431.3 mg/kg bw/day, respectively (pages 29 to 30 of the original submission).

<u>Fucose</u>

The study by Pitt et al. (2019) tested the safety of 3-FL preparation (94.6% 3-FL) containing 1.2% fucose. The results from the bacterial reverse mutation assay, mammalian micronucleus test, and *in vivo* mammalian erythrocyte micronucleus test, as well as an acute toxicity and a 90-

day subchronic toxicity studies in rats, support the safety of 3-FL and fucose present in HMO preparations (Pitt et al., 2019). Dietary subchronic exposure of rats to 3-FL (5 and 10%) did not produce any treatment-related abnormalities, and the NOAELs were placed at 5,980 and 7,270 mg/kg bw/day for male and female rats, respectively. The NOAELs for fucose may correspond to approximately 72 and 87 mg fucose/kg bw/day, respectively.

APTech's 2'-FL does not contain fucose. Thus, fucose is not included in APTech's new specifications for 2'-FL. Even if it would contain less than 1% of fucose, the estimated mean and 90th percentile EDIs are within the safe intake levels due to similar reasons discussed for 3-FL: the intended use would result in fucose concentration at 0.024 g/L in ready-to-drink or reconstituted formula prepared from powder. This would result in the estimated mean and 90th percentile intakes of 2.6 and 4.3 mg/kg bw/day, respectively.

FG

APTech has not detected any FG from its 2'-FL preparations, and FG is not a common HMO. Thus, APTech believes that it is not necessary to include FG in its specifications and have not included FG in the new specifications list.

References

Aldredge DL, Geronimo MR, Hua S, Nwosu CC, Lebrilla CB, Barile D. Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. Glycobiology. 2013;23(6):664-76.

FDA. 2020. GRN 000925. 3-fucosyllactose (Pending). Available at: https://www.fda.gov/media/142187/download

Parschat K, Oehme A, Leuschner J, Jennewein S, Parkot J. A safety evaluation of mixed human milk oligosaccharides in rats. Food Chem Toxicol. 2020;136:111118.

Pitt J, Chan M, Gibson C, Hasselwander O, Lim A, Mukerji P, Mukherjea R, Myhre A, Sarela P, Tenning P, Himmelstein MW, Roper JM. Safety assessment of the biotechnologically produced human-identical milk oligosaccharide 3-Fucosyllactose (3-FL). Food Chem Toxicol. 2019;134:110818.

Thurl S, Munzert M, Boehm G, Matthews C, Stahl B. Systematic review of the concentrations of oligosaccharides in human milk. Nutr Rev. 2017;75(11):920-33.

Question 4

4. In their October 23, 2020 amendment, the notifier states, that the parent strain Escherichia coli strain K12 MG1655 is available from the American Type Culture Collection (ATCC) as ATCC 70926 (page 22). However, in GRN 000932 and in several places in the notifier's October 23, 2020 amendment, E. coli strain K12 MG1655 is listed as available from ATCC as ATCC 700926 (e.g., page 13 of GRN 000932). Please provide a statement that corrects this reference on page 22.

APTech's response

The correct ATCC number for *Escherichia coli* strain K12 MG1655 is ATCC700926. ATCC70926 on page 22 of October 23, 2020 amendment was a typo. We apologize for causing the confusion.

We hope that the information above responds fully to FDA's follow-up questions regarding GRAS Notification 000932. We would be happy to provide you with any further information you may need.

Sincerely,

Susan S. Cho, Ph.D.
Susanscho1@yahoo.com
(301)875-6454