JHeimbach LLC

#897

November 21, 2019

Susan J. Carlson, Ph.D., Director
Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Dear Dr. Carlson:

Pursuant to 21 CFR Part 170, Subpart E, DuPont Nutrition and Health, through me as its agent, hereby provides notice of a claim that the addition of 2'-*O*-fucosyllactose produced by fermentation with *E. coli* K12 strain MG1655 INB000846 to non-exempt infant formula, toddler formula, infant and toddler foods, toddler drinks, conventional foods, and formula for oral and enteral tube feeding is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because DuPont has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

As required, one copy of the GRAS monograph and one signed copy of the conclusion from each member of the Expert Panel are provided. Additionally, I have enclosed a virus-free CD-ROM with the GRAS monograph and the signed statements of the Expert Panel.

If you have any questions regarding this notification, please feel free to contact me at 202-320-3063 or jh@jheimbach.com.

Sincerely,

[redacted]

James T. Heimbach, Ph.D., F.A.C.N.
President

Encl.

923 Water Street #66, Port Royal Virginia 22535, USA
tel. (+1) 804-742-5543 cell (+1) 202-320-3063 jh@jheimbach.com

Generally Recognized as Safe (GRAS) Determination for the Use of 2'-*O*-Fucosyllactose in Term Infant Formulas, Toddler Formulas, Foods Targeted to Toddlers, Conventional Foods, and Enteral and Oral Tube Feeding Formulas

Prepared by
JHeimbach LLC
Port Royal, Virginia

Prepared for
DuPont Nutrition and Health
Wilmington, Delaware

November, 2019

Table of Contents

List of Tables

List of Figures

Part 1. Signed Statements and Certifications

1.1. GRAS Notice Submission

DuPont Nutrition and Health (DuPont) of Wilmington, Delaware, through its agent James T. Heimbach, submits this GRAS notification in accordance with subpart E of 21 CFR part 170.

1.2 Name and Address of Notifier

DuPont Nutrition and Health
DuPont Experimental Station – E320
200 Powder Mill Road
Wilmington DE 19803

Contact: Jayne Davies
Tel: 302-695-6743
Email: jayne.c.davies@dupont.com

1.3. Name of Notified Substance

The subject of this Generally Recognized as Safe (GRAS) notice is 2'-*O*-fucosyllactose (2FL) produced by fermentation with *Escherichia coli* K12 strain MG1655 INB000846 (referred to as *E. coli* INB000846 in this document). A previous GRAS notice, GRN000749, which addressed 2FL produced by *E. coli* INB3051, is incorporated by reference. Both production strains are derived from *E. coli* K12 strain MG1655 through the insertion of the same four coding DNA fragments in the same locations, but strain INB000846 also includes insertion of an *E. coli* gene encoding lactose permease. The minor modification of the production organism to obtain strain INB000846 is described in this notice.

1.4. Intended Conditions of Use

The intended use of 2FL produced by *E. coli* INB000846 includes those foods included in GRN000749 (non-exempt infant formula, toddler formula, infant and toddler foods, and toddler drinks) as well as conventional foods and formula for oral and enteral tube feeding. 2FL produced by *E. coli* INB000846 is intended for addition to these foods as a nutrient supplement as described in 21 CFR §170.3(o)(20) to increase the dietary intake of 2'-*O*-fucosyllactose.

1.5. Statutory Basis for GRAS Status

DuPont Nutrition and Health has concluded that the intended use of 2FL produced by *E. coli* INB000846 is GRAS based on scientific procedures in accordance with 21 C.F.R. 170.30(b). This conclusion was made in concert with a panel of experts who are qualified by scientific training and experience.

Determination of the safety and GRAS status of the intended use of 2FL produced by *E. coli* INB000846 has been made through the deliberations of a GRAS Panel consisting of Joseph F. Borzelleca, Ph.D., Robert J. Nicolosi, Ph.D., and Michael W. Pariza, Ph.D., who reviewed a monograph prepared by James T. Heimbach, Ph.D., and other information deemed appropriate for this safety evaluation. These individuals are qualified by scientific training and experience to

evaluate the safety of food ingredients. They independently critically reviewed and evaluated the publicly available information and the potential human exposure to 2FL produced by *E. coli* INB000846 anticipated to result from its intended use, and individually and collectively determined that no evidence exists in the available information on 2FL produced by *E. coli* INB000846 that demonstrates, or suggests reasonable grounds to suspect, a hazard to consumers under the intended conditions of use of 2FL produced by *E. coli* INB000846.

It is the opinion of the GRAS Panel that other qualified scientists reviewing the same publicly available data would reach the same conclusion regarding the safety of the substance under its intended conditions of use. Therefore, the intended use of 2FL produced by *E. coli* INB000846 is GRAS by scientific procedures.

1.6. Premarket Exempt Status

Since DuPont Nutrition and Health has concluded that the intended use of 2FL produced by *E. coli* INB000846 is GRAS, such use is not subject to premarket approval requirements under the Federal Food, Drug, and Cosmetic Act.

1.7. Data Availability

The data and information that serve as the basis for the conclusion that the intended use of 2FL produced by *E. coli* INB000846 is GRAS will be made available to the FDA upon request. At FDA's option, a complete copy of the information will be sent to FDA in either paper or electronic format, or the information will be available for review at the offices of DuPont Nutrition and Biosciences, DuPont Experimental Station E320, 200 Powder Mill Road, Wilmington DE 19803 during normal business hours.

1.8. Freedom of Information Act Statement

No data or information submitted with this GRAS notification is exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552.

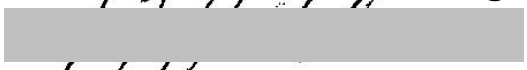
1.9. Certification

To the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the intended use of 2FL produced by *E. coli* INB000846.

1.10. FSIS Statement

Not applicable.

1.11 Name, Position, and Signature of Notifier



James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
Agent to DuPont Nutrition and Health

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

2.1. Name of the GRAS Substance

The subject of this Generally Recognized as Safe (GRAS) notice is 2'-*O*-fucosyllactose produced by fermentation with *Escherichia coli* K12 strain INB000846. GRAS notice GRN000749, which addressed 2FL produced by *E. coli* K12 strain INB3051, is incorporated by reference.

2'-*O*-fucosyllactose is frequently abbreviated as 2'-FL, 2-FL, or 2FL; the last of these is frequently used in this document. The IUPAC name is α-D-fucopyranosyl-(1→2)-β-D-galacto-pyranosyl-(1→4)-D-glucopyranose. The Chemical Abstracts Service (CAS) Registry number is 41263-94-9. The molecular weight is 488.44 Da and the empirical formula is $C_{18}H_{32}O_{15}$; the structural formula is shown in Figure 1.

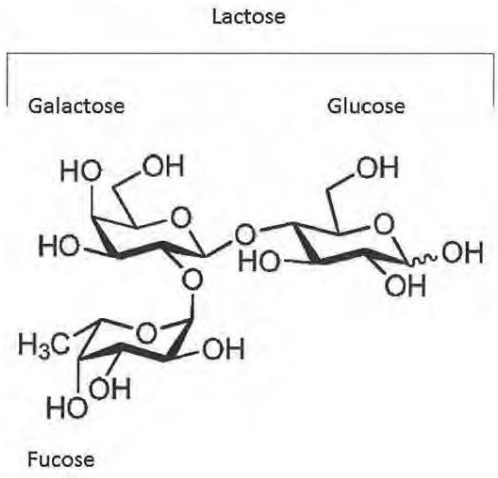


Figure 1. Structural Formula of 2FL.

Further information regarding the physical and chemical characteristics of 2FL is provided in GRN000749 and incorporated by reference. GRN000749 also notes that 2FL produced by fermentation with *E. coli* strain K12 INB3051 is chemically and structurally identical to analytical grade 2FL and to GRAS 2FL preparations that were the subjects of GRN000546, GRN000571, and GRN000650, to which FDA had no questions.

2.2. Source of the GRAS Substance

The 2'-O-fucosyllactose addressed in this notice is produced by fermentation of lactose and sucrose by *E. coli* strain INB000846. GRAS notice GRN000749, which is incorporated by reference, addresses 2FL produced by *E. coli* strain K12 INB3051. GRN000749 describes the derivation of strain INB3051 from parent strain *E. coli* strain K12 MG1655, a non-recombinant strain available from the American Type Culture Collection as ATCC70926 and from the Coli Genetic Stock Center as CGSC#7740. The parent strain is fully described, including the rationales on which it is considered not to be pathogenic or toxicogenic.

GRAS notice GRN000749 further describes the genetic manipulations that produced strain INB3051 from parent MG1655, including gene deletions, the sources of the 4 inserted genes, and the final transformation, as well as the strain's stability and safety.

Like strain INB3051, strain INB000846 (the production strain for the 2FL that is the subject of the current GRAS notice) is derived from *E. coli* strain K12 MG1655 using methods substantially identical to those used to derive strain INB3051. Four DNA insertions are the same as were used to produce INB3051:

- Gene *HpFutC*, origin *H. pylori*, encoding for fucosyltransferase
- Gene *BaSP*, origin *B. adolescentis*, encoding for sucrose phosphorylase
- Gene *ZmFrk*, origin *Z. mobilis*, encoding for fructokinase
- Gene *EcCscB*, origin *E. coli* W, encoding for sucrose transporter

And a fifth insertion that was not used in creating strain INB3051:

- Gene *EcLacY*, origin *E. coli* K12 MG1655, encoding for lactose permease

Insertion of the gene *EcLacY* into the production host increases the concentration of lactose permease, which is already naturally present in the host cell membrane. As a membrane-bound protein, lactose permease is insoluble in water. At the end of the fermentation, when the 2FL product stream passes through microfiltration and/or ultrafiltration membranes specifically selected to remove cell biomass and large molecules (including proteins and endotoxins), lactose permease is removed. As the mass of lactose permease is about 100-fold larger than 2FL (about 45,000 vs. 488 Da), the microfiltration and/or ultrafiltration steps provide reasonable certainty that lactose permease is not present in the finished 2FL product. (This step is illustrated in the post-fermentation process flow diagram that was presented in GRN000749 and is incorporated by reference.)

Specification of the origins of the inserted genes merely indicates that these genes are observed in these species; the inserted genes were not isolated from any donor strain but were synthesized *in vitro*. As was the case with strain INB3051, strain INB000846 includes artificial promoters and terminators to drive the new coding sequences as well as several small remnants ("DNA scars") left from gene knock-out and integration constructions.

As was the case with strain INB3051, as described in GRN000749, disruptions were engineered in genes that would interfere with the metabolic pathway required to produce 2FL. In addition to the deletions described in GRN000749, a partial deletion was made of *yhcE* and full deletions were made of *yhcG, yhcF* (putative proteins), and *yegH* (putative transport protein).

The stability of strain INB000846 was assessed through 61 generations of fermentation, through which the strain proved to be 100% stable.

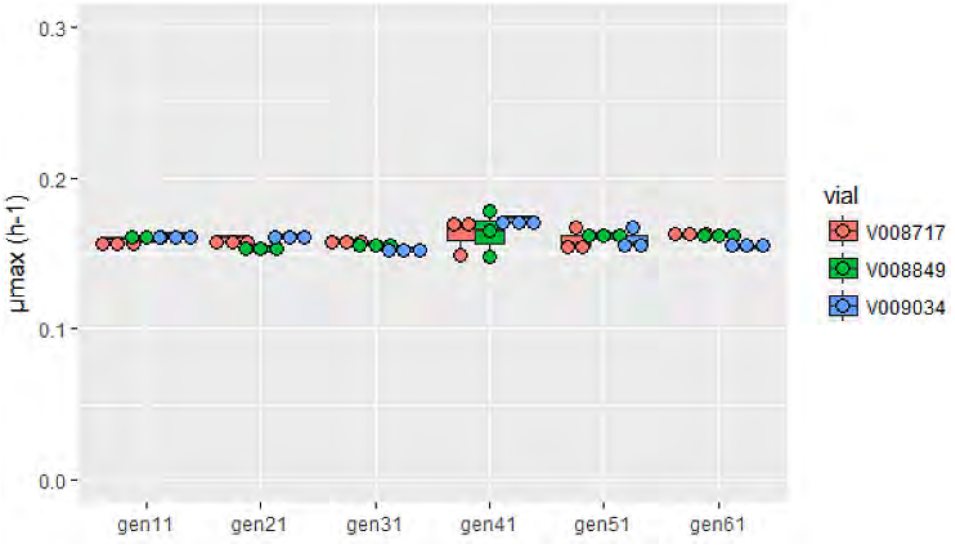


Figure 2. Growth Rate Assessment of Three Vials over 61 Generations.

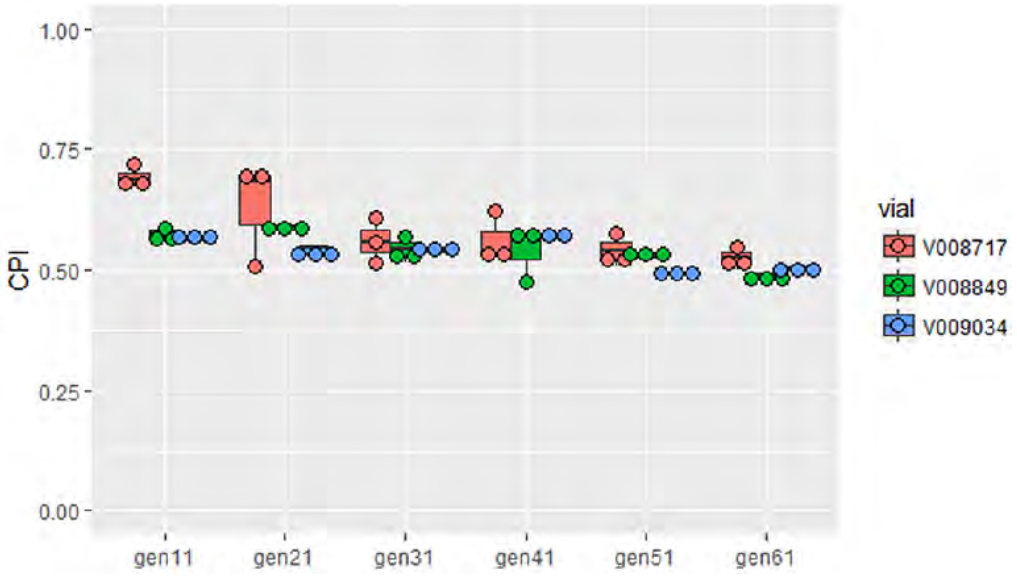


Figure 3. 2FL Production Assessment of Three Vials over 61 Generations.

Through all 61 generations no changes were reported in growth rate or production of 2FL, and full-genome sequences of samples taken at generations 11, 31, and 61 were identical. The strain produces a high level of 2FL, and the expressed proteins are *E. coli* lactose permease (*lacY*) and those also expressed by strain 3051 as described in GRN000749: *E. coli* anion symport for sucrose gene, *B. adolescentis* sucrose phosphorylase, *Z. mobilis* fructokinase, and a codon optimized version of *H. pylori* α-1,2-fucosyltransferase.

Strain INB000846 was deposited in the Inbiose culture collection.

2.3. Production Method

The production method is unchanged from the description provided in GRN000749, incorporated by reference.

2.4. Comparison of 2FL Derived from Strain INB000846 and Human Milk

One batch of 2FL produced by *E. coli* strain INB000846 was compared with a sample isolated from human milk by use of nuclear magnetic resonance spectroscopy (^{1}H NMR). As shown in Figure 4, all major well-resolved signals in the spectra of the two samples of 2FL are identical, indicating that there is no significant difference between 2FL derived from strain INB000848 and 2FL found in human milk.

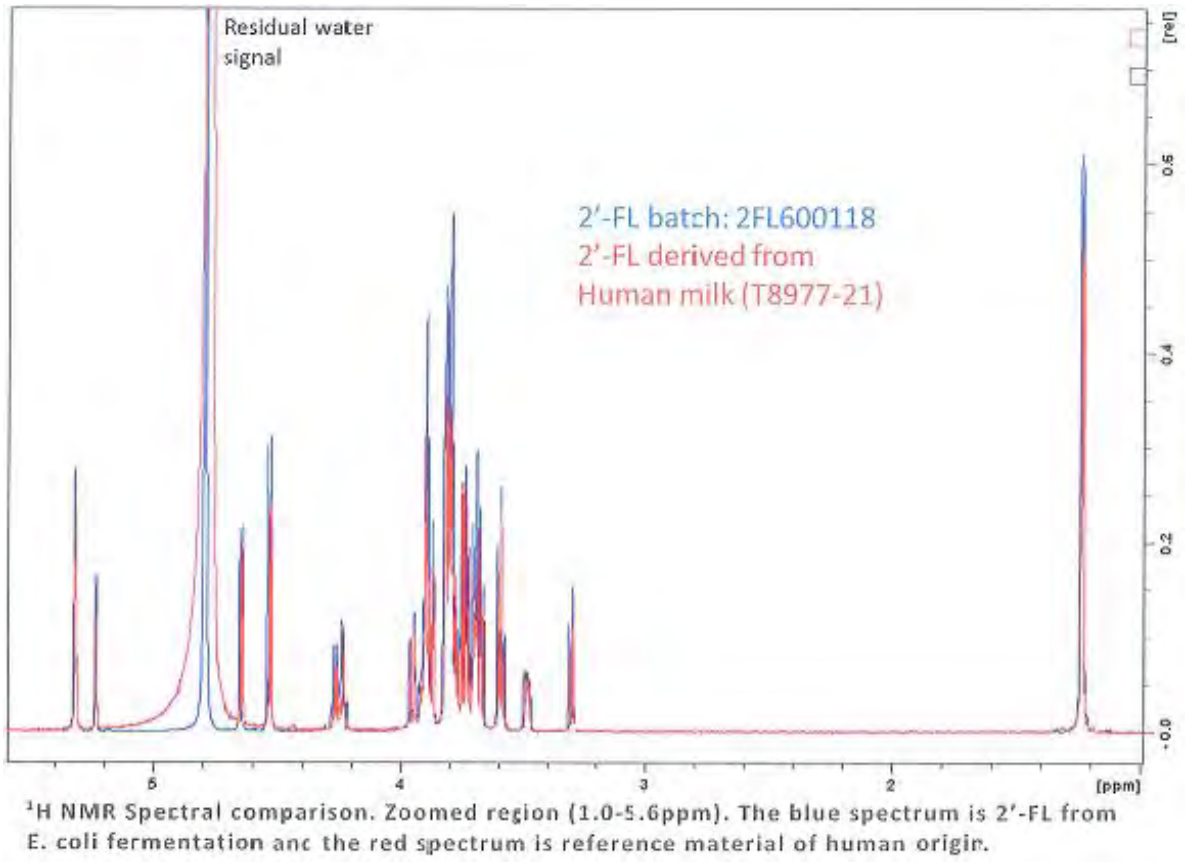


Figure 4. ^{1}H NMR Comparison of *E. coli*-Derived and Human-Derived 2FL.

2.5. Specifications

DuPont has established specifications for food-grade 2FL, displayed in Table 1. The results of analyses of three non-consecutive batches, also displayed in Table 1, show that the batches all conform with specifications, confirming that the production process is in control and is capable of consistently producing food-grade product.

Table 1. Specifications of 2FL.

Parameter	Specification	Tested Batches		
		2FL600118	2FL600218	2FL600318
Appearance				
Color	White/off-white	Pass	Pass	Pass
Form	Dry powder	Pass	Pass	Pass
In solution	≤300 ICUMSA[1]	28 ICUMSA	32 ICUMSA	12 ICUMSA
Chemical				
Moisture	≤5%	3.6%	4.0%	3.8%
Protein	≤100 µg/g	<1.39 µg/g	2.93 µg/g	3.64 µg/g
Total ash	≤0.5%	0.02%	0.02%	0.01%
Arsenic	≤0.2 mg/kg	<0.015 mg/kg	<0.015 mg/kg	<0.015 mg/kg
Cadmium	≤0.05 mg/kg	<0.001 mg/kg	<0.001 mg/kg	0.008 mg/kg
Lead	≤0.05 mg/kg	<0.007 mg/kg	<0.005 mg/kg	0.006 mg/kg
Mercury	≤0.1 mg/kg	<0.001 mg/kg	<0.001 mg/kg	<0.001 mg/kg
Endotoxins	≤300 EU/g	≤300 EU/g	≤300 EU/g	≤300 EU/g
GMO detection	Negative	Negative	Negative	Negative
Carbohydrate				
2FL	>96% (AUC[2])	97.8%	98.8%	98.1%
Lactose	<5% (AUC)	1.7%	0.9%	1.2%
Di-fucosyllactose	<5% (AUC)	0.6%	0.3%	0.6%
Other CHO	<5% (AUC)	0.0%	0.0%	0.0%
Microbial				
Standard plate count	<1000 cfu[3]/g	<100 cfu/g	<100 cfu/g	<100 cfu/g
Aerobic contaminants	<5000 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Total yeast and mold	<100 cfu/g	<20 cfu/g	<20 cfu/g	<20 cfu/g
Enterobacteriaceae	Not detected in 10 g	Pass	Pass	Pass
Salmonella spp.	Not detected in 750 g (30 x 25 g)	Pass	Pass	Pass
Listeria monocytogenes	Not detected in 25 g	Pass	Pass	Pass
Cronobacter sakazakii	Not detected in 300 g (30 x 10 g)	Pass	Pass	Pass
Coag+ staphylococci	<10 cfu/g (5 x 1 g)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)
Clostridium perfringens	<10 cfu/g (5 x 1 g)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)
Bacillus cereus	<10 cfu/g	<10 cfu/g	<10 cfu/g	<10 cfu/g
Enterococci	<100 cfu/g	<50 cfu/g	<50 cfu/g	<50 cfu/g
Clostridia spores	<10 cfu/g (5 x 1 g)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)

1. ICUMSA = International Commission for Uniform Methods of Sugar Analysis
2. AUC = area under the curve
3. cfu = colony-forming unit

2.6. Stability of 2FL

The results of accelerated stability studies in which three non-consecutive batches of 2FL were held at 40°C, 75% relative humidity, for 26 weeks showed no significant change in the content of 2FL, other carbohydrates, or microbiological parameters. These studies were fully described and reported in GRN000749 and are incorporated by reference.

Part 3. Intended Use and Dietary Exposure

2'-*O*-fucosyllactose produced by *E. coli* INB000846 is intended for addition to those foods included in GRN000749 (non-exempt infant formula, toddler formula, infant and toddler foods, and toddler drinks) and conventional foods and formulas for oral and enteral tube feeding as a nutrient supplement as described in 21 CFR §170.3(o)(20) to increase the dietary intake of 2'-*O*-fucosyllactose. Its effects include both nutrient value supporting metabolic processes and prebiotic effects serving as a substrate for commensal colonic bacteria. Estimated daily intakes (EDI) of 2FL produced by *E. coli* INB000846 are presented for these two categories of use.

3.1. EDI of 2FL in Infant and Toddler Formula, Infant and Toddler Foods, and Toddler Drinks

Table 2 displays the intended use of 2FL produced by *E. coli* INB000846 in non-exempt infant formula and toddler foods. This table is repeated for convenience from GRN000749, which (as noted previously) is incorporated by reference.

Table 2. Intended Use of 2FL in Infant and Toddler Formulas, Foods, and Drinks.

Intended Use	Reference Amount Customarily Consumed	Intended Use Level	
		g/RACC	g/kg (solids) g/L (liquids)
Non-exempt infant formula	100 mL	0.24	2.4
Toddler formula	100 mL	0.24	2.4
Infant & toddler foods	7-750 g	0.84 – 2.04	12
Toddler drinks	120 mL	0.14	1.2

GRN000749 observes that these uses and 2FL addition levels are the same as those set forth in Glycom's GRN000546 and GRN000650 and Jennewein's GRN571 and so result in no addition to the intake of 2FL by infants and toddlers; i.e., the DuPont product is an alternative to products already on the market. The estimated daily intakes of 2FL from uses in infant and toddler formulas, foods, and drinks, based on data from NHANES 2009-2012, are displayed in Table 3, adapted from Table 9 in GRN000749.

Table 3. 2FL Intakes by Consumers of Target Foods.

Source	Age Group (months)	g/person/day		mg/kg bw/day	
		Mean	90th Percentile	Mean	90th Percentile
Non-exempt infant formula	0-6	2.02	2.91	332.8	535.6
	7-12	1.70	2.63	188.9	295.8
	13-36	1.08	1.41	89.3	117.1
All intended infant and toddler food uses	0-6	2.93	5.29	449.7	712.4
	7-12	4.63	8.36	520.2	987.1
	13-36	1.12	1.97	84.9	146.0

3.2. EDI of 2FL in Conventional Foods and Tube-Feeding Formula

Table 4 displays the intended use of 2FL produced by *E. coli* INB000846 in conventional foods and tube-feeding formulas, including the category and descriptor of food, the NHANES summary, and the maximum intended use level.

Table 4. Intended Use of 2FL in Conventional Foods and Tube-Feeding Formula.

Food Category	Food Descriptor	NHANES Summary	Maximum Intended Use Level (g/kg)
Baked goods and baking mixes	Cereal and nutrition bars	Cereal bars, nutrition bars, & meal-replacement bars	30
Nonalcoholic beverages and beverage bases	Enhanced or fortified waters; Energy, sports & isotonic drinks & mixes	Enhanced & fortified waters, regular & low-calorie sport drinks & energy drinks	1.2
Breakfast cereals	Hot cereals	Oatmeal, cream of rice, cream of wheat, cream of rye, whole wheat hot cereal, oat bran hot cereal, grits, cornmeal mush	31
	RTE cereals	All types of RTE cereals	40
Milk products	Fermented & flavored milk, RTD & mixes	Buttermilk, kefir, flavored milk, hot chocolate, milk shakes, malted milk drinks	1.2
	Dairy & non-dairy smoothies & meal replacement beverages	Fruit & vegetable smoothies; meal replacement beverages such as Carnation Instant Breakfast, Muscle Milk, Slim Fast, & high protein drinks	5
	Yogurt	Regular & Greek yogurt, all flavors, excluding frozen yogurt	12
Dairy product analogs	Fluid milk substitutes	Soy milk, almond milk, rice milk, coconut milk (excluding coconut milk/cream used for cooking)	1.2
	Non-dairy yogurts	Soy & coconut milk yogurt	12
Processed fruits and fruit juices	Fruit juices, drinks, & nectars	100% fruit juices (excluding lemon juice), fruit & vegetable juice drinks, nectars, & coconut water	1.2
Processed vegetables and vegetable juices	Vegetable juices	100% vegetable juices	1.2
Tube-feeding formulas	Nutrient in enteral & oral tube-feeding formulas for patients ≥ 11 years	Nutritional beverages such as Boost, Ensure, and Glucerna as surrogates for tube-feeding formulas	20

The estimated daily intake (EDI) of 2'-*0*-fucosyllactose from the intended use of 2FL produced by *E. coli* INB000846 in food and tube-feeding formulas was calculated based on food consumption records collected in the What We Eat in America component of the NHANES conducted in 2013-2014 and 2015-2016 (WWEIA/NHANES 2013-2016). Two-day average intakes of all respondents aged 3+ years were estimated per user and expressed per person and on a bodyweight basis (Table 5).

Table 5. 2FL Intakes by Consumers of Target Foods.

Population	Per User Intakes (g/day)		Per User Intakes (mg/kg bw/day)	
	Mean	90th Percentile	Mean	90th Percentile
Children 3-12 y	1.6	3.5	60	140
Adolescents 13-18 y	1.7	3.9	30	60
Adults 19-49 y	2.2	5.2	30	70
Adults 50+ y	2.5	5.9	30	80
Total U.S. 3+ y	2.2	5.0	40	100

Overall, 81% of NHANES respondents consumed one or more of the intended-use food categories on at least one of the two survey days. The 90th percentile estimated intake of 2FL produced by *E. coli* INB000846 by the U.S. population is 5 g/day, equivalent to 100 mg/kg bw/ day. This is approximately double the intake per person of infants from the use of 2FL in non-exempt infant formula, but much lower than infants' intake in mg/kg bw/day.

Part 4. Self-Limiting Levels of Use

There is no meaningful limitation to the level of 2FL that can be added to foods or beverages.

Part 5. Experience Based on Common Use in Food

The GRAS conclusion is based on scientific procedures and not on common use in food before 1958. Nevertheless, naturally occurring 2FL from human breast milk has a long history of safe consumption. Additionally, infant formula supplements with 2FL have been on the market in the U.S. since 2016 with a history of safe consumption. Human milk exposure was extensively discussed in GRN000749, which is incorporated by reference, and is not repeated here. The GRN000749 discussion also includes the regulatory history of 2FL synthesized from benzyl-2-fucosyllactose (GRN000546), derived from fermentation with *E. coli* BL21 (GRN000571), and derived from fermentation with *E. coli* K12 (GRN000650), all of which were reviewed by FDA with no questions.

Part 6. Narrative

GRN000749, incorporated by reference, includes discussion of the history of safe ingestion of 2FL and the record of animal and human studies supporting the safety of such ingestion. These discussions are not repeated here. The remainder of this section discusses pertinent animal and human research published since preparation of GRN000749

6.1. Toxicity Studies

The safety of 94% pure 2FL produced by Friesland Campina using a genetically engineered strain of *E. coli* K12 (designated GI724/ATCC 55151) was evaluated by van Berlo et al. (2018) in a bacterial reverse mutation assay compliant with OECD test guideline No. 471, an *in vitro* mammalian cell micronucleus test compliant with OECD No. 487, and a subchronic oral toxicity study in rats compliant with OECD No. 408.

The bacterial reverse mutation test was performed by the plate incorporation method with *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537, and *E. coli* strain WP2 *uvrA*. 2FL was incorporated at levels of 0, 62, 185, 556, 1667, and 5000 µg/plate with and without metabolic (S9) activation. In both the absence and presence of S9-mix, 2FL did not induce a more than 2-fold and/or dose related increase in the mean number of revertant colonies compared to the background spontaneous reversion rate observed with the negative control. The potential clastogenic and aneugenic effects of 2FL were assessed in the *in vitro* mammalian cell micronucleus test with human peripheral blood lymphocytes exposed to 2FL at concentrations of 0, 500, 1000, or 2000 µg/mL with and without S9. No statistically significant, dose-dependent increase in the number of binucleated cells containing micronuclei was reported when compared to the concurrent solvent cultures in either the pulse treatment (with and without metabolic activation) or the continuous treatment (without metabolic activation). The number of binucleated cells containing micronuclei was within the test facility's historical data range of all respective control groups. The authors concluded that, “2’-fucosyllactose tested negative in both the bacterial reverse mutation test and the *in vitro* micronucleus test and should thus be considered as non-genotoxic” (van Berlo et al., 2018).

For the 90-day feeding study, 16 time-mated female Wistar Han IGS rats gave birth to 169 pups, 82 males and 87 females, from which 40 pups of each sex were randomized to 4 experimental groups (van Berlo et al, 2018). Experimental diets were prepared by adding 2FL to VRF1 cereal-based rodent diet at levels of 0, 3, 6, and 10% (w/w), and provided to the rats from postnatal day 25 to day 115. Fresh batches of feed were prepared each month and analyzed for stability, homogeneity, and concentration. At the outset, males weighed 52.2-75.8 g (mean = 64.4 g) while females weighed 48.2-71.7 g (mean = 60.0 g). Animals were housed 5 rats/cage with feed and water provided *ad libitum*.

Animal condition and behavior were monitored twice daily. Detailed clinical observations outside the cage were performed weekly. During week 12, a Functional Observation Battery (FOB) and a motor activity assessment were performed in all animals. Ophthalmoscopic changes were assessed in the last week of the exposure period in animals from the control and high-dose groups. (Because no treatment-related ocular changes were reported in the high-dose group, eye examination was not extended to the animals in the lower dose groups.) Feed and water consumption from each cage were measured twice weekly and bodyweights were recorded weekly. At necropsy, blood was collected from the abdominal aorta for analyses of

clinical chemistries[1] and hematology[2]; urine was collected for urinalysis[3] and examination of sediment. Macroscopic examination was performed at necropsy and organ weights were determined[4]. Tissue samples[5] were excised and preserved and all samples from the control and high-dose group were subjected to histopathological examination. (Since no treatment-related changes were reported in the high-dose group, histopathology was not extended to the other groups.)

The overall mean intake of 2FL was 2.17, 4.27 and 7.25 g/kg bw/day for males and 2.45, 5.22 and 7.76 g/kg bw/day for females from the low-, mid-, and high-dose group, respectively. No exposure-related mortality or clinical signs were reported and the results of the detailed clinical observations, FOB, and motor activity assessment did not indicate any neurotoxic potential of 2FL. In female rats of the high-dose group, feed consumption was statistically significantly decreased, but there was no difference among feeding groups for male rats and bodyweights were not different in either sex. There were no statistically significant differences in clinical chemistry variables between the test groups and the controls except for an increase in urea concentration in mid- and high-dose males. In the absence of this finding in females and any corroborative findings in males, this was considered as a chance finding. There were no statistically significant differences in red blood cell, total white blood cell, or differential white blood cell variables between the test groups and the controls. A statistically significantly decreased urine density was reported in high-dose females. The decreased density was only slight and ascribed to a higher (although not statistically significant) urinary volume excreted. Because these changes were slight, they do not point to impaired concentrating ability of the kidneys and no toxicological significance was attached to this finding. The relative weight of the liver was statistically significantly increased in high-dose males. The absolute and relative weights of the filled and empty cecum were statistically significantly increased in the mid- and high-dose group in male and female rats, an effect ascribed to the fact that the test article is a non-digestible carbohydrate. At necropsy, no exposure-related macroscopic changes were reported. Microscopic evaluation did not reveal exposure-related histopathological changes.

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[1] ALP, AST, ALT, GGT, total protein, albumin, urea, creatinine, glucose, bilirubin, total cholesterol, triacylglycerol, phospholipids, Ca, Na, K, Cl, and inorganic P.

[2] Hemoglobin, packed cell volume, red blood cell count, reticulocytes, total white blood cell count, differential white blood cell counts, thrombocyte count, prothrombin time, mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.

[3] Volume, appearance, density, pH, glucose, occult blood, ketones, total protein, bilirubin, and urobilinogen.

[4] Adrenals, brain stem, cerebrum, cerebellum, cecum, epididymides, heart, kidneys, liver, ovaries, prostate, seminal vesicles, spleen, testes, thymus, and uterus.

[5] Adrenals, aorta, axillary lymph nodes, brain, cecum, colon, epididymides, esophagus, eyes, gut-associated lymphoid tissue, heart, kidneys, liver, lungs, mammary gland, mesenteric lymph nodes, ovaries, oviducts, pancreas, parathyroid, parotid salivary glands, pituitary, prostate, rectum, sciatic nerve, seminal vesicles, skeletal muscle, skin, duodenum, ileum, jejunum, spinal cord, spleen, sternum, stomach, sublingual salivary glands, submaxillary salivary glands, testes, thymus, thyroid, trachea/bronchi, urinary bladder, uterus, vagina and any tissue showing gross lesions.

The authors reported that "the exposure to 2FL was well tolerated at all dose levels, and did not induce any relevant changes in general condition, growth, water intake, neurobehavioral observations, ophthalmoscopy, hematology, clinical chemistry, urinalysis, organ weights or in macroscopy and microscopy of organs and tissues." Based on these conclusions, the no observed adverse effect level (NOAEL) was placed at the highest level tested, 7.25 g/kg bw/day for males and 7.76 g/kg bw/day for females (van Berlo et al., 2018).

Phipps et al. (2018) studied the safety of an 8:1 mixture of 2FL and difucosyllactose in *in vitro* genotoxicity testing and a subchronic study of oral toxicity in neonatal rats, both conducted in compliance with Good Laboratory Practice and OECD guidelines (OECD No. 471 and 487 for genotoxicity, OECD No. 408 for 90-day rodent studies). The authors suggested that, since these two oligosaccharides are always found together in human milk, a mixture of the two (in the proportions occurring in human milk) would better simulate the oligosaccharide fraction of human milk. The mixture tested was produced by Glycom A/S using microbial fermentation.

Genotoxicity testing included a bacterial reverse mutation test using *Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537, and *E. coli* strain WP2 *uvrA*, which were exposed to the oligosaccharide mixture at concentrations of 5, 15, 50, 150, 500, 1500, or 5000 µg/plate with and without S9 activation; all tests were conducted in triplicate. An *in vitro* mammalian cell micronucleus test was conducted using the cytokinesis-block method with human peripheral blood lymphocytes exposed to the mixture at concentrations of 500, 1000, or 2000 µg/mL for 3 hours in the absence and presence of S9 and for 20 hours in its absence.

In the bacterial reverse mutation test, there were no biologically relevant differences in the mean number of revertant colonies following exposure to the oligosaccharide mixture, compared with exposure to the vehicle control in either the absence or presence of metabolic activation. The micronucleus test produced no evidence of clastogenicity or aneugenicity in the absence of any biologically relevant differences in the percentage of micronucleated cells between oligosaccharide-exposed cultures and vehicle controls. The authors concluded that, "The results of the *in vitro* genotoxicity tests demonstrated that 2'-FL/DFL [the 8:1 mixture of 2FL and difucosyllactose] is non-genotoxic."

The 90-day gavage study of oral toxicity deviated from OECD No. 408 in beginning test-article administration at 7 days of age—during weaning rather than after weaning. This variation aligned the dosing period with that described in guidelines for safety assessment of compounds intended for pediatric application (FDA 2006; EMEA 2008; MHLW 2012). Twenty female Crl:CD®(SD) time-mated rats provided 15 randomized litters of pups, which were pooled on postnatal day 2 and randomly redistributed to provide litters of 6 pups/sex. On postnatal day 4, litters were culled to 5 animals/sex and assigned to dose groups, each group being assigned 2 litters (i.e., 10 pups/sex/group). The dose groups were vehicle control; 1000, 3000, or 5000 mg/kg bw/day; and reference group receiving 5000 mg fructooligosaccharides (FOS)/kg bw/day. The daily gavage provided a constant dose volume of 10 mL/kg bw/day. The authors reported that doses were selected based on a preliminary 14-day study in which no test-article-related effects were observed in neonatal rats dosed at 4000 or 5000 mg/kg bw/day from postnatal day 7.

An additional 5 males and females were included in the vehicle control, high-dose, and FOS reference groups to constitute recovery groups to be retained for 4 weeks after dosing. Pups were housed with their dams until weaning on postnatal day 21, then in 5 animals/cage with free access to feed and water. Formulations were prepared weekly; those from the first and last weeks

were analyzed for accuracy and homogeneity. Observations for morbidity and mortality were made twice daily, detailed physical examinations were performed daily on dosing days 1-14 and weekly thereafter. Observations for clinical signs associated with dosing took place before, immediately after, and 1-2 hours after dosing. Ophthalmic observations were made of animals in the control, high-dose, and reference groups during the last week of dosing. Animals were weighed daily for 2 weeks and then twice weekly and feed consumption was recorded twice weekly. All animals were examined to determine the ages of eye opening, emergence of air-righting reflex, and attainment of sexual maturity. Startle response and pupil-closure response were evaluated on dosing day 16; a FOB was conducted on all animals during week 11 of dosing; and performance in a Morris water maze was assessed during week 12. At the end of dosing, blood was collected from the sublingual vein for analysis of clinical chemistry, hematological, and coagulation parameters. Urine was collected for analysis of clarity color, pH, specific gravity, bilirubin, blood pigments, total protein, creatinine, and glucose. All animals were subjected to full macroscopic necropsy, selected organs were weighed, and selected organs and tissues were fixed for histopathological examination; these examinations were conducted only for animals in the vehicle control and high-dose groups while tissues from animals in the other groups were retained but not examined.

At the beginning of dosing, males weighed 13.1-20.9 g and females weighed 11.9-19.2 g. There were no deaths and no test item-related clinical signs or ocular findings and no differences among groups in feed consumption, bodyweight gain, ulna length, age of sexual maturation or air-righting reflex, or performance on the Morris maze. High-dose females showed significantly lower mean activity counts compared with controls, but this was not reported in males and there was no dose-response relationship; it was thus considered to be unrelated to the test article. Statistically significant differences from control values were reported in a number of hematological and coagulation parameters (white blood cell count, red blood cell count, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, prothrombin time, and concentrations of lymphocytes, monocytes, eosinophils, and basophils), but these were not consistent across sex, were not associated with dose-response, and were all within the normal historical ranges; they were not regarded as test-article related. Similarly, statistically significant variations in blood chemistries (AST, albumin, creatinine, blood urea nitrogen, calcium, and phosphorus) were observed in only one sex or did not show dose-response and were all within normal range, and thus were not test-article related. Minor differences in urinalysis values were also considered to be biologically irrelevant and unrelated to administration of the test article because they were reported only in females and were not accompanied by differences in physical, biochemical, or microscopic urinary parameters. Absolute organ weights did not differ significantly, and significantly increased relative kidney and seminal vesicle weights were seen only in low-dose males and thymus weights in all males; with no indication of dose relationships, these differences were regarded as incidental. Finally, macroscopic and microscopic evaluation at necropsy "only revealed findings that were incidental and commonly observed in rats of this age and strain."

The authors noted that, "values for high-dose 2'-FL/DFL animals were generally similar to those for animals receiving FOS (reference control), an ingredient already approved as safe for use in infant formula." They concluded that "5000 mg/kg bw/day (the highest dose tested) was

established as the NOAEL[6]" (Phipps et al., 2018). The authors also observed that, "The absence of any genotoxicity or subchronic toxicity associated with administration of a combination of 2'-FL and DFL is consistent with the fact that both compounds are endogenous in human and mammalian milks"

The authors went on to suggest:

> Traditionally, an MoE [Margin of Exposure] of 100 between the NOAEL and anticipated exposure is attempted to be established for food additives occurring at low levels in food; however, interpretation of the MoE must take into account sub-populations such as infants, where intakes relative to body weight are much higher compared with adults and infant formula is the only source of nutrition for the first 12 weeks of life, with exposure limited to that relatively short period of time. Furthermore, it is not possible to administer macronutrients such as 2′-FL/DFL to animals at the levels required to meet this criterion, due to formulation limitations and the potential for nutritional imbalances at excessively high doses. Taking these issues into account, it has been concluded that an MoE between 1 and 10 is acceptable for infants under 12 weeks of age consuming the food additive in infant formula (JECFA, 2014). A lower MoE is also considered acceptable as HMOs are not absorbed to a significant degree and there are no structural alerts for mutagenicity" (Phipps et al., 2018).

6.2. Other Animal Studies

A number of other studies have been published in which 2FL was given to animals, usually to evaluate potential benefits. While safety was not an endpoint in these studies, the absence of adverse effects from ingestion of 2FL supports the conclusion of safety reached based on toxicity studies.

Using ^{13}C-labeled 2FL, Kuntz et al. (2019) studied the metabolic fate and distribution of 2FL in a murine model. Forty 8-week-old male NMRI mice weighing 36-47 g were gavaged with 1 g ^{13}C-2FL/kg bw (n = 5) or saline (n = 3) through the tail vein. Animals were kept in metabolic cages for collection of urine and feces and sacrificed after 0.5, 1, 2, 3, 5, 9, or 15 hours; small and large intestine, brain, liver, heart, spleen, and kidney were removed. A similar experiment was conducted with 12 6-week-old male C3H/HeN germ-free mice weighing 29-35 g.

2FL was primarily eliminated in the feces. ^{13}C-enrichment in plasma and in brain and other organs showed a maximum peak after 5 hours, but was only detected when the ^{13}C-2FL bolus reached the colon. In germ-free mice, the ^{13}C-bolus remained in the intestinal content and was expelled in the feces. The authors suggested that, "after the application of ^{13}C-labelled 2FL, ^{13}C-fucose, or a fucose metabolite carrying the ^{13}C-label, most likely generated by the intestinal microbiota, was responsible for the ^{13}C-enrichment in the systemic circulation and organs, as opposed to intact 2FL." They concluded that 2FL itself does not reach the systematic circulation and direct incorporation of 2FL in the brain and other organs does not appear to be required to produce reported effects.

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[6] Since the test article was an 8:1 mixture of 2FL and difucosyllactose, the NOAEL for 2FL alone was 8/9 of this level, or 4444 mg/kg bw/day.

Azagra-Boronat et al. (2018) studied the effect of 2FL produced by microbial fermentation (>90% purity) and other oligosaccharides on rotavirus-induced diarrhea in suckling rats. Fifteen G15 Lewis rats gave birth to litters that were culled to 8 pups each; dams received chow and water *ad libitum*. Three dams and their 24 pups were assigned to each of 5 groups:

- Reference group
- Rotavirus-infected (RV) control group
- RV group supplemented with 2 g 2FL/kg bw/day
- RV group supplemented with 8 g galacto- and fructo-oligosaccharides/kg bw/day
- RV group supplemented with 2 g 2FL, and 8 g GOS + FOS/kg bw/day

Treatments were administered by gavage daily from day 2 to day 16 of life; the rotavirus was inoculated at day 5. Bodyweight was recorded and fecal samples were taken daily. Half (n = 12) of each group of rats was sacrificed at day 8, the peak of diarrhea, and the rest at day 15. Thymus, spleen, liver, and intestines were weighed and small intestine samples were examined histologically and for permeability as well as for concentrations of IL-1α, IL-4, IL-6, IL-10, IL-12p70, IFN-γ, and TNF-α. Cecal contents were analyzed for short-chain fatty acids and blood samples were analyzed for IgM, IgG1, IgG2a, IgG2b, IgG2c, and IgA.

Both 2FL and GOS/FOS significantly reduced the incidence, severity, and duration of diarrhea. The authors reported that 2FL promoted intestinal maturation and enhanced neonatal immune response, while the effect of GOS and FOS was due to intestinal trophic effect. 2FL, but not GOS/FOS, ameliorated much of the cytokine production boosted by the rotavirus and decreased the secretion of the proinflammatory cytokines IL-1b, IL-6, IL-12, IFN-g, and TNFa. No histopathological lesions were reported from ingestion of 2FL and no adverse effects were reported.

Grabinger et al. (2019) reported on the effects of 2FL, 3-fucosyllactose (3FL), 3-sialyllactose (3SL), and 6-sialyllactose (6SL) on the course of intestinal inflammation in a murine model, interleukin-10 null (*Il10*$^{-/-}$) mice after weaning. Mice (number, sex, and bodyweights not reported) were weaned on postnatal day 21 and given a 5 mM solution of 2FL, 3FL, 3SL, 6SL, of D-lactose in water available *ad libitum* for 26 days. The intestinal microbiota was depleted by administration of vancomycin, ampicillin, and neomycin in drinking water and metronidazole by gavage for 6 days between the 4th and 6th week of life. After sacrifice, tissue from the proximal and distal colon were subjected to histopathological analysis for colitis, isolation of RNA, and quantitative polymerase chain reaction for DNA.

The analysis of inflammatory markers, cytokines, and markers of epithelial integrity in the distal colon of mice after 4 weeks of oligosaccharide supplementation revealed that 2FL, but not the other tested oligosaccharides, led to significantly decreased expression of the pro-inflammatory markers iNOS, IL-1b and IL-6 and significantly increased expression of TGFβ, a factor involved in wound healing, and occluding, a tight-junction protein associated with epithelial integrity. The anti-inflammatory effect of 2FL supplementation on colitis in *Il10*$^{-/-}$ mice was confirmed by the observation that only 2FL supplementation maintained a normal colon length in the mice and decreased diarrhea and intestinal permeability. Blind scoring of histological sections confirmed that supplementation with 2FL, but not 3FL, reduced colitis. No adverse effects were reported associated with 2FL or the other oligosaccharides.

The ability of a mixture of 2FL (>90% purity, produced by bacterial fermentation), short-chain GOS, and long-chain FOS to modulate the gut microbiota, improving the efficacy of

vaccine-induced immunity, was reported by van den Elsen et al. (2019) in a murine model. Breeding pairs of BALB/c mice (numbers not reported) were fed control diet from the day of mating or a diet supplemented with a 2% admixture of the oligosaccharide mixture introduced at mating, at birth of pups, or at weaning. Mice were vaccinated with trivalent influenza vaccine at 6 weeks, and both development of the gut microbiota and antibody-mediated vaccine responses were followed over time. All animals were retained on their assigned diet (control or prebiotic) until sacrifice 90 days after the vaccination.

Prebiotic diet consumption during pregnancy did not alter the litter size of the breeding pairs. There were no visible differences in the health of offspring born from breeding pairs fed control or 2FL/GOS/FOS diet and body weights remained similar throughout the study. Female mice demonstrated a larger antibody response to the vaccination than males, but the prebiotic diet improved vaccine-specific antibody response in male mice. No adverse effects in either sex were reported associated with the 2FL/GOS/FOS supplement.

6.3. Human Studies

Kajzer et al. (2016) reported a prospective, randomized, double-blind, controlled 3-arm, multi-center study with 88 singleton term infants (sex not reported) with birth weights ≥2490 g. Infants were enrolled before postnatal day 8 and assigned to receive experimental formula with 0.2 g/L 2FL and 2 g/L FOS (n = 46) or without oligosaccharide (n = 42) until postnatal day 35. A human-milk reference group (n = 43) was also enrolled. Formula intake, stooling patterns, anthropometric measures, and parental reports of any adverse effects were tracked.

The study was completed by 86% and 89% of infants in the experimental and control groups, respectively. No differences were reported between the formula groups in formula intake, growth, stool frequency or consistency, or adverse effects. The authors concluded that the "formula containing 2FL and scFOS was well tolerated in young infants as evidenced by stool consistency, formula intake, anthropometric data and percent feedings with spit-up/vomit similar to that of infants fed formula without oligosaccharides or human milk."

In an assessment of infant tolerance for 2FL in formula, Storm et al. (2019) enrolled 78 healthy 2-week-old (mean age = 14±3.3 days) singleton term infants (45M, 33F) in a prospective, randomized, double-blind, placebo-controlled, multi-center trial. Both the test and control infant formulas contained protein in the form of 100% partially hydrolyzed whey and 10^6 cfu *B. animalis* ssp. *lactis* strain Bb12/g powder, but the test formula also contained 259 mg 2FL/L. At enrollment, anthropometric measures were taken and the Infant Gastrointestinal Symptom Questionnaire (IGSQ[7]) was administered. Infants consumed their assigned formulas (n = 38 test formula, n = 40 control formula) *ad libitum* for 42 days before returning for anthropometric measurement and re-administration of the IGSQ.

During the final 2 days of the feeding period, caregivers recorded the amount of formula consumer, the number of stools and consistency of each stool, difficulty in stooling, frequency of spitting up or vomiting, and frequency and duration of crying and fussing. Adverse events (AEs)

[7] The IGSQ is a validated 13-item questionnaire that assesses an infant's GI-related signs and symptoms as observed by caregivers/parents over the previous week in 5 domains: stooling, spitting up/vomiting, flatulence, crying, and fussing

were collected throughout the study and were assessed by the site investigator or designee for duration, intensity, frequency, and relationship to the test product. AEs were classified into System, Organ, and Class categories. Within the infections and infestations System, Organ, and Class category, 5 AE clusters were identified: upper respiratory tract infection, viral upper respiratory tract infection, otitis media/ pharyngitis, thrush, and "other."

In both the test and control groups, 1 subject was lost to follow-up, 1 caregiver wished to withdraw, and 3 withdrew due to adverse events. In the control group, 2 caregivers were in noncompliance and discarded from the analysis. The primary outcome was comparison of IGSQ scores after 6 weeks of feeding; there was no significant difference between the feeding groups. Among secondary outcomes, no difference was reported in stool frequency or consistency, defecation difficulty, spit-up, vomiting, crying, or fussing. There were no differences in formula consumption or anthropometric measures. With regard to AEs:

> "There were no serious AEs reported in the study. Seventy-two AEs occurred in the study, 36 in the Test group and 36 in the Control group, corresponding to 17 and 19 subjects in the Test and Control groups, respectively. Spit-up reported as an adverse event was of interest due to the finding that there were more subjects with spit-up noted as "frequent" in the Test group compared with the Control group; however, only one subject in each group reported "mild" spit-up as an AE, and no subjects had reports of more extreme spitting up. In the category of reported infections and infestations, there were more subjects with this category of AE in the Control versus the Test Group (Control 9 [23%] vs Test 3 [8%], P = .05). A P value of .05 is marginally significant and suggestive of a possible association between 2′FL and the lower rate of infections. However, the small number of cases experiencing infections suggests interpreting this P value with caution. Looking specifically at upper respiratory infections, there was a higher but nonsignificant incidence in the Control 4 (10%), versus Test 0 (0%), P = .12. Overall, there were no safety concerns noted with either of the study formulas" (Storm et al., 2019).

The authors concluded, "An infant formula with 100% whey, partially hydrolyzed, as the protein source with the addition of 0.25 g/L of the HMO 2'FL and probiotic *B lactis* is tolerated well based on a comprehensive tolerance assessment tool and is tolerated similarly to an otherwise identical formula without 2'FL."

Nowak-Wegrzyn et al. (2019) reported on a prospective, randomized, double-blind, placebo-controlled, crossover trial assessing the tolerance of infants with documented cow's milk protein allergy for a whey-based extensively hydrolyzed formula with or without 1.0 g 2FL and 0.5 g LNnT/L. A total of 67 infants were enrolled—45 males and 22 females aged 2-57 months (mean age = 24.5±13.6 months) and first underwent food challenges with each formula, in which infants under 1 year of age ingested formula in doses of 5 ml, 10 ml, 20 ml, 30 ml, 30 ml, 35 ml, and 50 ml (total 180 ml) at 10-15 minute intervals, while the doses for infants >1 year were 5 ml, 10 ml, 25 ml, 45 ml, 45 ml, 45 ml, and 65 ml (total 240 ml). Any allergic signs or symptoms (cutaneous, gastrointestinal, respiratory, or cardiovascular) attributable to the challenge formula were documented. All infants passing the challenge test received the 2FL/LNnT formula for 1 week, consuming at least 240 ml daily. Formula intake; daily stool frequency, color, consistency, and odor; flatulence; spitting-up or vomiting; any potential allergic symptoms; and any other adverse events were recorded.

Sixty-one infants completed the challenge with both formulas; all drop-outs were for protocol violations. Only one infant reacted during the challenge, showing urticaria and

erythematous rash in response to both formulas. During the 7-day consumption of the test formula with 2FL and LNnT, 2 patients had reported gastrointestinal symptoms—one vomited on day 1 but had no further problems and the other developed diarrhea on day 7, attributed to gastroenteritis, which resolved after 4 days. The authors indicated that, "Otherwise, no significant gastrointestinal symptoms (flatulence, abnormal stool frequency/consistency, increased spitting-up or vomiting) were reported. There were no reactions that warranted early discontinuation of the open formula challenge. No serious adverse events occurred during the entire study."

6.4. Safety Assessment and GRAS Determination

6.4.1. Introduction

This section presents an assessment that demonstrates that the intended use of 2'-*O*-fucosyllactose produced by *E. coli* INB000846 is safe, and is GRAS.

This safety assessment and GRAS determination entail two steps. In the first step, the safety of the intended use of 2FL produced by *E. coli* INB000846 is demonstrated. Safety is established by demonstrating that the likely intake of the substance under its intended conditions of use is within allowable levels of intake. In the second step, the intended use of 2FL produced by *E. coli* INB000846 is determined to be GRAS by demonstrating that the safety of this substance is generally recognized among qualified scientific experts and is based on publicly available and accepted information.

The regulatory framework for establishing whether a substance is GRAS, in accordance with Section 201(s) of the Federal Food Drug and Cosmetic Act, is set forth under 21 CFR §170.30. This regulation states that general recognition of safety may be based on the view of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. A GRAS determination may be made either: 1) through scientific procedures under §170.30(b); or 2) through experience based on common use in food, in the case of a substance used in food prior to January 1, 1958, under §170.30(c). This GRAS determination employs scientific procedures established under §170.30(b).

A scientific procedures GRAS determination requires the same quantity and quality of scientific evidence as is needed to obtain approval of the substance as a food additive. In addition to requiring scientific evidence of safety, a GRAS determination also requires that this scientific evidence of safety be generally known and accepted among qualified scientific experts. This "common knowledge" element of a GRAS determination consists of two components:

1) Data and information relied upon to establish the scientific element of safety must be generally available; and

2) There must be a basis to conclude that there is a consensus among qualified experts about the safety of the substance for its intended use.

The criteria outlined above for a scientific procedures GRAS determination are applied below in an analysis of whether the intended use of 2FL produced by *E. coli* INB000846 is safe, and is GRAS. Once the intended use is determined to be GRAS, it is permitted to be used as intended, because it is by definition not a food additive and therefore does not require promulgation of a food additive regulation under 21 CFR prior to being marketed and sold in the United States.

A scientific procedures GRAS determination requires that information about the substance establish that the intended use of the substance is safe. The FDA has defined "safe" or "safety" for food additives under 21 CFR §170.3(i) as "a reasonable certainty in the minds of competent scientists that the substance is not harmful under its intended conditions of use." This same regulation specifies that three factors must be considered in determining safety. These three factors are:

> 1) the probable consumption of the substance and of any substance formed in or on food because of its use (i.e., the EDI);
>
> 2) the cumulative effect of the substance in the diet, taking into account any chemically- or pharmacologically-related substance or substances in such diet; and
>
> 3) safety factors which, in the opinion of experts qualified by scientific training and experience to evaluate the safety of food and food ingredients, are generally recognized as appropriate.

6.4.2. Estimated Daily Intake

The estimated daily intake of 2FL produced by *E. coli* INB000846 by infants and toddlers from its intended use in non-exempt infant formula (2.4 g/L), toddler formula (at 2.4 g/L), toddler drinks (at 1.2 g/L), and infant and toddler foods (at 12 g/kg) was reported in GRN000749, where it was noted that this intake is the same as that from previously notified GRAS determinations and so does not represent an increase in exposure. The highest mean and 90th–percentile intakes from non-exempt formula are by infants aged up to 6 months, 2.02 and 2.91 g/person/day, respectively (equivalent to 332.8 and 535.6 mg/kg bw/day, respectively). The highest mean and 90th–percentile intakes from all intended infant and toddler food uses are 4.63 and 8.36 g/person/day (520.2 and 987.1 mg/kg bw/day) by 7-12-month-olds.

The estimated daily intake of 2FL produced by *E. coli* INB000846 from its intended use in conventional foods (with use levels of 1.2 g/L in beverages and ranging up to 40 g/kg in RTE cereals) and enteral and oral tube-feeding formulas (with use level = 20 g/L) was estimated based on the WWEIA component of NHANES 2013-2016. The highest mean and 90th-percentile estimated per-person intakes of 2FL are by adults aged 50 years and older, 2.5 and 5.9 g/person/day, respectively. However, on a body-weight basis, the highest estimated intakes are by children aged 3-12 years, with mean and 90th-percentile intakes of 60 and 140 mg/kg bw/day, respectively. For the total U.S. population aged 3 years and older, the 90th–percentile estimated intake is 5 g/day, equivalent to 100 mg/kg bw/ day.

It should be noted that, while the per-person intakes of 2FL by older children and adults are higher than those of infants and children, the highest intakes on a body-weight basis are by infants under the age of 12 months, with intakes (mg/kg bw/day) nearly 10 times higher than those of older children and adults.

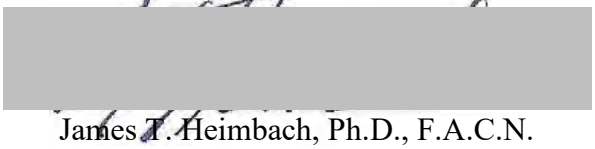
6.5.3. Safety

The safety and GRAS status of the use of 2FL in non-exempt infant formula, toddler formula, toddler drinks, and infant and toddler foods was substantiated in GRN000749 and incorporated by reference. The safety and GRAS status of the use of 2FL in conventional foods and in enteral and oral tube-feeding formulas is well supported on several grounds:

1. The estimated daily intake of 2FL, on a body-weight basis, is only about 10% of the intake of infants from the uses described in GRN000749
2. Published OECD-compliant studies by Phipps et al. (2018) and van Berlo et al. (2018) demonstrated that 2FL is non-genotoxic.
3. High-quality OECD-compliant subchronic oral toxicity studies by Phipps et al. (2018; gavage study) and van Berlo (2018; feeding study) showed that 2FL is non-toxic, with both NOAELs set at the highest doses tested, 7.25 g/kg bw/day for males and 7.76 g/kg bw/day for females in van Berlo et al. (2018) and 5.0 g/kg bw/day for both sexes in Phipps et al. (2018). These NOAELs are about twenty times higher than the mean intake of infants from non-exempt infant formula and about 150 times higher than intakes from the use of 2FL in conventional foods and in enteral and oral tube-feeding formulas.
4. Several published randomized clinical trials support the safety of 2FL, including Kajzer et al. (2016) and Nowak-Wegrzyn et al. (2019). Of particular importance is Storm et al. (2019), which partook of some of the elements of a Phase-II clinical trial with extensive assessment of safety and adverse events. No 2FL-associated adverse events were reported in any of these studies.

6.5. Statement Regarding Information Inconsistent with GRAS

I have reviewed the available data and information and am not aware of any data or information that are, or may appear to be, inconsistent with our conclusion of GRAS status of the intended use of 2FL.



James T. Heimbach, Ph.D., F.A.C.N.

Part 7. List of Supporting Data and Information

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Generally Recognized as Safe (GRAS) Determination for the Use of 2'-*O*-Fucosyllactose in Term Infant Formulas, Toddler Formulas, Foods Targeted to Toddlers, Conventional Foods, and Enteral and Oral Tube Feeding Formulas

Conclusion of the GRAS Panel

Determination of the safety and GRAS status of 2FL produced by *E. coli* INB000846 under its intended conditions of use has been made through the deliberations of a GRAS Panel consisting of Joseph F. Borzelleca, Ph.D., Robert J. Nicolosi, Ph.D., and Michael W. Pariza, Ph.D. We are qualified by scientific training and experience to evaluate the safety of food and food ingredients. We have critically reviewed and evaluated the publicly available information summarized in the document *Generally Recognized as Safe (GRAS) Determination for the Use of 2'-O-Fucosyllactose in Term Infant Formulas, Toddler Formulas, Foods Targeted to Toddlers, Conventional Foods, and Enteral and Oral Tube Feeding Formulas*, prepared by James T. Heimbach, Ph.D., and dated November 2019, including the potential human intake resulting from the intended use of 2FL produced by *E. coli* INB000846, and have individually and collectively concluded:

> We unanimously conclude that the ingestion of 2'-*O*-fucosyllactose produced by *E. coli* INB000846 from its intended use results in a level of intake that is within safe limits established by the history of consumption of this substance and by published animal and human studies.
>
> We further conclude that, the use of 2'-*O*-fucosyllactose produced by *E. coli* INB000846, produced consistent with cGMP and complying with the specifications and use described in the GRAS monograph, is safe and GRAS based on scientific procedures.
>
> It is our opinion that other qualified and competent scientists reviewing the same publicly available information would concur with these conclusions.

Joseph F. Borzelleca, Ph.D. Date: 26 November 2019
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Robert J. Nicolosi, Ph.D. _____ Date: _____
Professor Emeritus
University of Massachusetts—Lowell
Lowell, Massachusetts

Michael W. Pariza, Ph.D. _____ Date: _____
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

Generally Recognized as Safe (GRAS) Determination for the Use of 2'-*O*-Fucosyllactose in Term Infant Formulas, Toddler Formulas, Foods Targeted to Toddlers, Conventional Foods, and Enteral and Oral Tube Feeding Formulas

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Joseph F. Borzelleca, Ph.D. _____ Date: _____
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Robert J. Nicolosi, Ph.D. _____ Date: 21 November 2019
Professor Emeritus
University of Massachusetts—Lowell
Lowell, Massachusetts

Michael W. Pariza, Ph.D. _____ Date: _____
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

Generally Recognized as Safe (GRAS) Determination for the Use of 2'-*O*-Fucosyllactose in Term Infant Formulas, Toddler Formulas, Foods Targeted to Toddlers, Conventional Foods, and Enteral and Oral Tube Feeding Formulas

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Joseph F. Borzelleca, Ph.D. _____ Date: _____
Professor Emeritus
Virginia Commonwealth University School of Medicine
Richmond, Virginia

Robert J. Nicolosi, Ph.D. _____ Date: _____
Professor Emeritus
University of Massachusetts—Lowell
Lowell, Massachusetts

Michael W. Pariza, Ph.D.  Date: November 20, 2019
Professor Emeritus
University of Wisconsin—Madison
Madison, Wisconsin

From:	jheimbach@va.metrocast.net
To:	Hice, Stephanie; jh@jheimbach.com
Cc:	"Lim, Angela"; "Jayne Davies"
Subject:	RE: GRN 000897 - Questions for Notifier
Date:	Thursday, April 2, 2020 9:27:42 AM
Attachments:	Hice Stephanie 20200402.pdf Questions 1-7.pdf Question 8.pdf Question 9.pdf

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Dear Dr. Hice:

We are responding to the questions you asked on March 20 concerning GRN 000897. We again wish to thank you for arranging the conference call that allowed us to achieve a better understanding of FDA's issues.

There are four pdf documents attached to this email:

- Cover letter addressed to you
- Responses to questions 1-7 (including a revision of Table 1 from the GRN)
- Response to question 8
- Response to question 9

Thank you for your patience.

Regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
JHeimbach LLC
923 Water Street #66
Port Royal VA 22535
USA
Tel: (+1) 804-742-5543
Cell: (+1) 202-320-3063
Email: jh@jheimbach.com

JHeimbach LLC

April 2, 2020

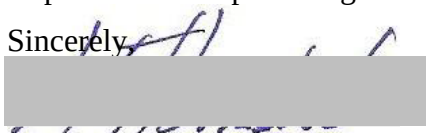
Stephanie Hice, Ph.D.
Staff Fellow (Biology)
Division of Food Ingredients
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
U.S. Food and Drug Administration

Dear Dr. Hice:

On March 20, 2020, you notified us that, during your review of GRN 000897, you noted a number of questions. We requested further clarification, and you very helpfully arranged for a conference call on March 25 during which we and several FDA reviewers discussed the issues for which you requested additional information.

Attached to this letter are our responses to the FDA questions. We believe that the responses we are providing will address these issues to your satisfaction.

Sincerely,



James T. Heimbach, Ph.D., F.A.C.N.
President

923 Water Street #66, Port Royal Virginia 22535, USA
tel. (+1) 804-742-5548 cell (+1) 202-320-3063 jh@jheimbach.com

Questions/Comments 1-7 Regarding GRN 000897:

1. *Please specify the intended source of the protein base (e.g., milk, soy, whey, etc.) of infant formula that 2'-FL will be added into.*

   Response: The intended sources of the protein base of the non-exempt infant formula include cow's milk and soybean.

2. *Please note that, while the United States does not have a definition for "toddler formula", the Agency recognizes it as formula intended for infants 12+ months of age. However, if it is intended for infants under 12 months of age (for example, 9-18 months) then these products must follow the infant formula regulations as the intended population includes infants less than 12 months of age.*

   Response: The notifier, referred to as DuPont, would like to clarify that in this notification "toddler formula" refers to formulas intended for infants and young children 12 months of age and older.

3. *On pages 3 and 13 of the notice, the notifier refers to 2'-FL as a "nutrient". 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.*
   Response: DuPont would like to clarify that the intended use of 2'-FL is as an ingredient. Accordingly, the referenced descriptors on pages 3 and 13 should be changed to "ingredient."

4. *Please clarify if internally-developed methods of analysis used for specification parameters have been validated for that particular purpose. If using standard methods, please provide appropriate citations.*
   Response: DuPont has primarily utilized standard methods for analysis of 2'-FL to demonstrate conformance with the stated specifications. The carbohydrate analysis is an exception and used an internally validated method for HILIC (Hydrophilic Interaction Liquid Chromatography). The use of standard methods for the other parameters prevents the need for method validation. We have attached a revised Table 1 that now specifies the method used for each specification parameter.

5. *Please clarify whether the provided specifications for Salmonella serovars in 2'-FL are performed using a 750-gram sample (page 10). If so, please provide results from analysis of three non-consecutive batches for Salmonella serovars in a sample size of 25 grams of 2'-FL.*

   Response: DuPont would like to clarify that the sample size for Salmonella servovars analysis was 25 grams. The data presented captures the analytical results of 30 individually analyzed samples within each of the 3 non-consecutive lots, a total of 90 samples, each of 25 grams. The table below includes a revised description of this specification.

6. *Please clarify whether the provided specifications for Cronobacter sakazakii in 2'-FL are performed using a 300-gram sample (page 10). If so, please provide results from analysis of three non-consecutive batches for C. sakazakii in a sample size of 10 grams of 2'-FL.*

Response: We would like to clarify that the sample size for Cronobacter sakazakii analysis was 10 grams. The data presented captures the analytical results of 30 individually analyzed samples within each of the 3 non-consecutive lots, a total of 90 samples, each of 10 grams. The table below includes a revised description of this specification.

7. *The notifier states that the intended use of 2'-FL is GRAS based on scientific procedures (21 CFR 170.30(b)), however, includes a discussion in Part 5, Experience Based on Common Use in Foods (page 16). Please note, that the information provided in Part 5 does not meet the regulatory definition of "Common Use in Foods" as defined by 21 CFR Part 170.245. We note, that the provided discussion should be incorporated into Part 6, Narrative, as defined by 21 CFR Part 170.250.*
Response: The information provided in the discussion in Part 5 will be moved to Section 6.4.2. Estimated Daily Intake.

Parameter	Specification	Method	Tested Batches		
			2FL600118	2FL600218	2FL600318
Appearance					
Color	White/off-white	visual	Pass	Pass	Pass
Form	Dry powder	visual	Pass	Pass	Pass
In solution	≤300 ICUMSA[1] units	ICUMSA Method GS $1_{/3}$-7	28 ICUMSA	32 ICUMSA	12 ICUMSA
Chemical					
Moisture	≤5%	Karl-Fischer titration	3.6%	4.0%	3.8%
Protein	≤100 µg/g	Bradford Assay	<1.39 µg/g	2.93 µg/g	3.64 µg/g
Total ash	≤0.5%	NMKL 173:2005	0.02%	0.02%	0.01%
Arsenic	≤0.2 mg/kg	EN 15763:2009	<0.015 mg/kg	<0.015 mg/kg	<0.015 mg/kg
Cadmium	≤0.05 mg/kg	EN 15763:2009	<0.001 mg/kg	<0.001 mg/kg	0.008 mg/kg
Lead	≤0.05 mg/kg	EN 15763:2009	<0.007 mg/kg	<0.005 mg/kg	0.006 mg/kg
Mercury	≤0.1 mg/kg	EN 15763:2009	<0.001 mg/kg	<0.001 mg/kg	<0.001 mg/kg
Endotoxins	≤300 EU/g	Ph. Eur. 2.6.14 + Interference study	≤300 EU/g	≤300 EU/g	≤300 EU/g
GMO detection	Negative	PCR (internally validated, EFSA 2018[3])	Negative	Negative	Negative
Carbohydrate					
2FL	>96% (AUC[2])	HILIC[4]	97.8%	98.8%	98.1%
Lactose	<5% (AUC[2])	HILIC[4]	1.7%	0.9%	1.2%
Di-fucosyllactose	<5% (AUC[2])	HILIC[4]	0.6%	0.3%	0.6%
Other CHO (calculated by dfference)	<5% (AUC[2])	HILIC[4]	0.0%	0.0%	0.0%
Microbial					
Standard plate count	<1000 cfu[5]/g	ISO 4833-1	<100 cfu/g	<100 cfu/g	<100 cfu/g
Aerobic contaminants	<5000 cfu/g	ISO 4833-1	<10 cfu/g	<10 cfu/g	<10 cfu/g
Total yeast and mold	<100 cfu/g	ISO 21527	<20 cfu/g	<20 cfu/g	<20 cfu/g
Enterobacteriaceae	Not detected in 10 g	ISO 21528-1	Pass	Pass	Pass
Salmonella spp.	Not detected in 25g (30 individual samples per batch i.e. 30x25g)	ISO 6579-1	Pass	Pass	Pass
Listeria monocytogenes	Not detected in 25 g	ISO 11290-1	Pass	Pass	Pass
Cronobacter sakazakii	Not detected in 10g (30 individual samples per batch i.e.30x10g)	ISO 22964	Pass	Pass	Pass
Coag+ staphylococci	<10 cfu/g (5 x 1 g)	ISO 6000-1	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)
Clostridium perfringens	<10 cfu/g (5 x 1 g)	ISO 7937	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)
Bacillus cereus	<10 cfu/g	ISO 7932	<10 cfu/g	<10 cfu/g	<10 cfu/g
Enterococci	<100 cfu/g	ISO 7899	<50 cfu/g	<50 cfu/g	<50 cfu/g
Clostridia spores	<10 cfu/g (5 x 1 g)	ISO 15213	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)

1. ICUMSA = International Commission for Uniform Methods of Sugar Analysis
2. AUC = area under the curve
3. EFSA 2018. Guidance on the characterization of microorganisms used as feed additives or production organisms; Section 3.2 – evaluation of fermentation products for presence of DNA from the production strain. EFSA Journal. 163:5206.
4. HILIC- Hydrophilic Interaction Liquid Chromatography
5. cfu = colony-forming unit

Q8: The notice includes intended use in enteral and tube-feeding formulas that was not described in previous GRNs for 2'-FL. Given that consumers of tube-feeding formulas constitute a vulnerable sub-population, and that the suitability of providing l- digestible carbohydrate in such formulas may be problematic (e.g., see Tarleton etal.,2013), please provide a narrative that supports the safe use of your ingredient for this intended use (at the maximum intended use level of 20 g/kg).

Response: The article cited by FDA (Tarleton SM, Kraft CA, DiBaise JK. 2013. Fiber-enriched enteral formulae: advantageous or adding fuel to the fire? *Practical Gastroenterol*, December:11-22.) observes that the addition of low-digestible carbohydrates (CHO) to enteral formulas is intended to normalize bowel function and improve feeding tolerance, but suggests that the presence of certain comorbidities may contraindicate such addition. The article also suggests that the possible benefits of addition of non-digestible carbohydrates may be less well supported than is generally supposed. Since GRAS is concerned with safety and tolerance rather than benefit, we will focus on the first point.

The authors advance only two medical disorders in which comorbidity may result in adverse safety or tolerance regarding the addition of low-digestible CHO to enteral feedings—patients at high risk for bowel ischemia or severe dysmotility. These are both easily observable conditions, and it is likely that the health professional overseeing the administration of partial or total enteral nutrition would be aware of the patient's status. The article's conclusion that "we recommend its [fiber's] judicious use," is a conclusion with which we concur.

Another (more recent) article brought to our attention by FDA takes a diametrically opposite position, calling for greater concern with <u>under</u>utilization of low-digestible CHO in enteral formula, especially elemental or peptide formula (O'Keefe SJD. 2018. The need to reassess dietary fiber requirements in healthy and critically ill patients. *Gastroenterol Clin North Am* 47:219-229). This author argues that enteral feeding "generally overlook the metabolic needs of the colon, and when combined with antibiotics may predispose patients to dysbiosis, bacterial overgrowth with pathogens such as *C. difficile*, and acute colitis". There is no discussion in this article with any risk of adverse effects due to excessive intake of such CHO, and it is clear that the author does not believe that such risks are significant.

There are no published studies documenting the safety/tolerability of the addition of 2'FL to enteral formulas so we rely upon the broader literature on the safety/tolerability of a wide range of low-digestible CHO. Research to date bears out the belief that risks of adverse effects from judicious addition of low-digestible CHO to enteral formula, while probably not zero, are well within the GRAS standard of relative certainty of no harm. In the table below are summaries of a number of randomized clinical trials and open-label studies in which non-digestible CHO were added to enteral feedings given to preterm infants, children, healthy adults, bed-ridden elderly adults, and patients hospitalized for a variety of serious medical conditions. The test articles include partially hydrolysed guar gum (PHGG), galactomannan, fructooligosaccharides (from scFOS to long-chain inulin), galactooligosaccharides, and GOS/FOS blends, with ingestion levels often greater than 20 g/day and as high as 63 g/day. No adverse effects were reported in any study. While no claim is made that this survey of the literature is exhaustive, it is not selective in choosing only supportive research.

Additionally, we cite the Institute of Medicine (IOM)'s 2005 review of potential adverse effects from overconsumption of fiber. Without repeating the specific conclusions, we simply note that the IOM regarded adverse effects as so unlikely that no necessity was seen for establishing a Tolerable Upper Intake Level.

Citation	Study Design	Subjects	Dose	Duration	Safety-Related Findings
Studies of PHGG in Enteral Feeding					
Lampe et al. (1992)	Prospective, randomized, double-blind, crossover study	11 healthy men	enteral formula providing 15 g PHGG/day	18 days	Fecal wet and dry weights, fecal moisture content, fecal pH, and stool frequency were decreased. Stool weight and fecal consistency did not change significantly and no adverse effects were reported. The authors concluded that, "despite significant differences in mean transit time, few differences in other parameters of bowel function were observed when healthy subjects consumed enteral formula diets containing 0 g of fiber and 15 g of total dietary fiber as modified guar and soy."
Meier et al. (1993)	Open-label study	12 healthy men	enteral formula supplemented with PHGG; intake 42 g PHGG/day	7 days	Significantly increased colonic but not orocecal transit time compared with either a self-selected diet or the enteral formula without fiber. PHGG had no effect on stool consistency or frequency.
Alam (1993)	Randomized double-blind cross-over study of PHGG	10 healthy adults	42 to 63 g/day of PHGG in enteral formula	1 week	No tolerance issues; hemoglobin, hematocrit, total and differential WBC count, Na, K, Mg, Cl, ALT, AST, γ-GT, alkaline phosphatase, bilirugin, creatinine were all within normal ranges.
Homann et al. (1994)	Prospective randomized double-blind placebo-controlled trial	100 hospital patients; 30 receiving total enteral nutrition and 70 receiving enteral supplementation	20 g PHGG/L of formula; intake of TPN patients = 24 g PHGG/day; intake of enteral supplementation patients = 20 g PHGG/day	Not reported	Those receiving either total or supplemental enteral nutrition had reduced incidence and severity of diarrhea but increased flatulence. No bloating or cramping was noted. 4 patients on the standard total enteral diet, but no patients receiving PHGG, had to be discontinued due to gastrointestinal side effects. In the supplemental feeding groups, 8 control v. 2 PHGG patients had to discontinue feeding. The authors reported that "The total number of GI-side effects was not different in the two groups (17 in each group)."

Citation	Study Design	Subjects	Dose	Duration	Safety-Related Findings
Fussell et al. (1996)	Prospective, randomized, double-blind, placebo-controlled study	57 tube-fed adults in 5 diagnostic categories: abdominal surgery/ trauma, cerebral trauma, head/neck surgery, multiple fractures, and vascular surgery	14 g PHGG/L of formula	5-14 days	No significant effect on diarrhea was observed, nor on albumin, transthyretin, or flatulence. The PHGG was generally well tolerated.
Peters and Davidson (1996)	Prospective, randomized, double-blind cross-over study	12 enterally fed patients with Type 1 diabetes	Not reported	Not reported	The 2 formulas containing PHGG (concentration not specified) were not effective in attenuating the postprandial glucose excursion, but no adverse effects were reported.
Spapen et al. (2001)	Prospective, randomized, double-blind, placebo-controlled study	25 ICU patients (13 M, 12 F; mean age = 68.5±13.1 years) with severe sepsis and septic shock fed enterally	22 g PHGG/L of formula	At least 6 days	The group receiving PHGG supplementation exhibited significantly reduced frequency of diarrhea and a reduction in the number of days with diarrhea; there was no significant effect on sepsis-related mortality (1 death in the test group, 4 in the control) or duration of stay in the intensive care unit. The authors concluded that “Fiber treatment was well-tolerated” and “Total enteral nutrition supplemented with soluble fiber is beneficial in reducing the incidence of diarrhea in tube-fed full-resuscitated and mechanically ventilated septic patients.”

Citation	Study Design	Subjects	Dose	Duration	Safety-Related Findings
Homann et al. (2004)	Prospective, randomized, double-blind, placebo-controlled trial	100 medical and surgical patients (50 patients per group); 30 patients received total enteral nutrition and 70 patients received 1000 ml/day supplemental enteral nutrition	20 g PHGG/L	Not reported	Use of PHGG resulted in significantly fewer patients with diarrhea (6 vs. 15 on the fiber-free formula) and a significant reduction in the number of days patients suffered from diarrhea (10.2 vs. 40.6 days). The number of patients experiencing GI side effects was the same in both groups (n = 17 per group), although flatulence was reported in more patients in the PHGG group. Enteral nutrition was discontinued due to GI side effects in 4 patients on the control diet, but no patients on the PHGG-supplemented diet.
Rushdi et al. (2004)	Prospective, randomized, double-blind, controlled study	20 IBS patients (11 M, 9 F; aged 28-73 years with mean age = 57/5±13/8 years) on enteral nutrition with 3 or more liquid stools/day	2% (22 g PHGG/L) 22 to 37 g PHGG/ day	4 days	Supplementation with PHGG significantly reduced the number of liquid stools. The PHGG was well tolerated with fewer adverse gastrointestinal symptoms than in the control group. The authors discussed tolerance issues extensively: "Throughout the course of this clinical trial, in the fiber-enriched feed group, only two patients complained of flatulence (20%). On the other hand, in the control group, four patients complained of flatulence (40%), two patients got vomiting (20%) and one case of constipation (10%) was reported. However, no statistical significance was found between both groups as regards incidence or severity of gastrointestinal symptoms. None of these symptoms was severe enough to necessitate therapeutic intervention."

Citation	Study Design	Subjects	Dose	Duration	Safety-Related Findings
Studies of Galactomannan in Enteral Feeding					
Nakao et al. (2002)	Open-label study	20 elderly bed-ridden males and females (10 M, 10 F, mean age = 79.3±5.1 years) receiving enteral feeding	7 g galacto-mannan/day during the first week; the dose was upped 7 g/day each week until they received 28 g galactomannan/ day for the fourth week	4 weeks	Serum diamine oxidase activity significantly increased. The water content of the feces decreased, and the frequency of normal stools increased. The frequency of bowel movements, number of aerobic bacteria, and the pH of feces decreased, while fecal SCFA, especially acetic and propionic acids, increased. All effects reversed after termination of the galactomannan supplementation. There was no change in counts of total bacteria or anaerobes and no change in body weight, total serum protein, prealbumin, transferrin, retinol-binding protein, total cholesterol, triacylglycerol, iron, copper, or zinc. No adverse effects were reported.
Studies of Fructooligosaccharides in Enteral Feeding					
Karakan et al. (2007)	Randomized, double-blind, placebo-controlled trial of adding scFOS to early enteral nutrition solution for feeding of patients with severe acute pancreatitis	30 patients aged 46.1±14.0 years with severe acute pancreatitis requiring stoppage of oral feeding	0 or 24 g fiber (about 50% scFOS)/day	2 days	The median durations of enteral feeding and of the hospital stay were significantly shorter in the group receiving the prebiotic. Significant improvement was also seen in pancreatitis severity scores and the authors reported that, “In conclusion, nasojejunal EN with prebiotic fiber supplementation in severe AP improves hospital stay, duration of nutrition therapy, acute phase response and overall complications compared to standard EN therapy.” Both enteral feeding solutions were well tolerated with no reported adverse effects.
Khoshoo et al. (2010)	Randomized, double-blind crossover trial of enteral formula with FOS	14 children aged 1-15 years receiving 75-100% of calories via feeding tube	3.5 g FOS/L; intake 3.5 g FOS/ day	2 weeks	There were no withdrawals; stools improved; no effect on vomiting, abdominal pain, or weight gain. “This study showed that a peptide-based formula containing fiber was as well-tolerated as a fiber-free formula in a small population of children with gastrointestinal impairments.”

Citation	Study Design	Subjects	Dose	Duration	Safety-Related Findings
Garleb et al. (1996)	Randomized, double-blind, controlled study of the effect of the addition of scFOS to enteral feeding formulas	27 apparently healthy male college students	0, 5, or 10 g scFOS/L; daily intakes of 0, 15, and 30 g scFOS	for 14 days	No change in body weight or deviations from the normal range of blood chemistry values. Fecal acetate, isobutyrate, and isovalerate concentrations were higher among students ingesting scFOS, but no differences in propionate or butyrate, fecal pH, or fecal percent dry matter. Consumption of scFOS increased fecal bifidobacteria. Tolerance of the scFOS-containing formula was good. Complaints of nausea, cramping, distension, vomiting, diarrhea, and regurgitation were similar across all groups and were present on fewer than 5% of participant-days. Flatus was reported more frequently by those consuming 30 g scFOS/day, but most complaints occurred during the first 4 days. The authors concluded that "these results indicate that [scFOS] does not compromise serum chemistry profiles, is well tolerated particularly at an intake of 15 g/d and would serve as a bifidogenic factor when incorporated into a liquid enteral product."
Karakan et al. (2007)	Randomized, double-blind, placebo-controlled trial of adding scFOS to early enteral nutrition solution for feeding of patients with severe acute pancreatitis(AP)	30 patients aged 46.1±14.0 years with severe acute pancreatitis requiring stoppage of oral feeding	0 or 24 g fiber (about 50% scFOS)/day	2 days	The median durations of enteral feeding and of the hospital stay were significantly shorter in the group receiving the scFOS. Significant improvement was also seen in pancreatitis severity scores and the authors reported that, "In conclusion, nasojejunal EN with prebiotic fiber supplementation in severe AP improves hospital stay, duration of nutrition therapy, acute phase response and overall complications compared to standard EN therapy." Both enteral feeding solutions were well tolerated with no reported adverse effects.

Citation	Study Design	Subjects	Dose	Duration	Safety-Related Findings
Simakachorn et al. (2011)	Randomized, double-blind, placebo-controlled study of tolerance of critically ill children for an experimental enteral formula	47 critically ill children age 1-3 years under mechanical ventilation and enteral feeding	2.6 g/L of oligo-fructose/inulin and 2.8 g/L of acacia gum in combination with 2 strains of live microorganisms	7 days	Abdominal distension, vomiting, and stool frequency were unaffected. Concluded that the experimental enteral formula is safe and well tolerated by children in intensive care receiving enteral nutrition.
Studies of Galactooligosaccharides or GOS/FOS in Enteral Feeding					
Modi et al. (2010)	Prospective, randomized, double-blind, placebo-controlled multi-center trial	77 preterm infants (GA <33 weeks) receiving enteral feeding	8 g/L of scGOS/ lc FOS in a 9:1 ratio	~8 weeks or until discharge	There was no overall difference in tolerance between control and supplemented formula, but addition of prebiotic to formula improved tolerance for the most immature infants. There were no differences in gains in weight, length, or head circumference; in stooling frequency, stool characteristics, or fecal microbiota; or in GI signs or water balance (based on concentrations of serum sodium and creatinine). The authors concluded that prebiotic supplementation is safe.
Akatsu et al. (2016)	Prospective, randomized, double-blind, placebo-controlled trial of immune effect of experimental formula in enterally-fed elderly patients	Enterally fed elderly individuals	GOS and new prebiotic-bifidogenic growth stimulator (BGS) via percutaneous endoscopic gastrostomy	10 weeks	No adverse effects reported.

Citation	Study Design	Subjects	Dose	Duration	Safety-Related Findings
Armanian et al. (2016)	Prospective, randomized, double-blind, placebo-controlled trial in preterm neo-nates with hyper-bilirubinemia	25 hyper-bilirubinemic preterm neonates who had reached 30 ml/kg bw/day enteral feeding volume	scGOS/lc FOS in a 9:1 ratio	1 week	No adverse effects were reported. The authors concluded that, "Prebiotic oligosaccharides increase stool frequency, improve feeding tolerance and reduce bilirubin level in preterm neonates and therefore can be efficacious for the management of neonatal hyperbilirubinemia."
Van den Berg et al. (2015)	Prospective, randomized, double-blind, placebo-controlled trial of immune effects in very preterm infants	113 infants with GA <32 weeks or birth-weight <1500 g	scGOS/lc FOS/ pectin-derived acidic oligo-saccharides(pAOS)	4 weeks	No AEs reported. Conclusion: "Enteral supplementation of scGOS/lcFOS/pAOS has a regulatory effect on the response to conjugated polysaccharide pneumococcal vaccine with normalization of the enhanced responses in preterm infants toward levels similar to healthy term infants."

IOM Panel on Macronutrients on Adverse Effects from Overconsumption of Fiber

To estimate the human requirement for fiber, the Panel on Macronutrients (IOM 2005) reviewed a large body of research relating fiber intake to a number of health endpoints. These included reduction in the risk of hyperlipidemia, hypertension, and coronary heart disease; gastrointestinal health, including duodenal ulcers, constipation, laxation, fecal weight, SCFA production, and diverticular disease; colon cancer, breast cancer, and other cancers; glucose tolerance and insulin response; and satiety and weight maintenance. The panel elected to use the level of fiber intake needed to achieve significant reduction in the risk of coronary heart disease as the basis for establishing a minimum human requirement.

Data were lacking to set an estimated average requirement (EAR) because the benefit of elevated total fiber intake occurs continuously across the whole range of intakes against which impact on the advent of coronary heart disease is now known from prospective studies (IOM 2005). The Panel noted:

> "Because the available evidence suggests that the beneficial effects of fiber in humans are most likely related to the amount of food consumed, not to the individual's age or body weight, the best approach is to set an Adequate Intake (AI) based on g/1000 kcal" (IOM 2005).

Based on the average of the reviewed studies on dietary fiber and coronary heart disease, and the beneficial role of functional fibers, the Panel set the AI for total fiber at 14 g/1000 kcal. The Panel considered that there is no reason to believe that fiber intake as a function of energy intake differs during the life cycle; thus, AIs for various sex/age groups were determined by multiplying [14 g/1000 kcal] X [median energy intake of each group].

The Panel on Macronutrients (IOM 2005) reviewed the published literature regarding the potential for adverse effects due to overconsumption of dietary fiber and due to overconsumption of functional fiber. One area of particular emphasis was the effect of fiber intake on mineral bioavailability, particularly calcium, magnesium, iron, and zinc. The panel concluded that there is little evidence that fiber itself, absent phytate, has adverse effects on mineral absorption or status. The panel also concluded that intake of dietary fiber at levels in excess of 40 g/day do not result in significant increases in gastrointestinal distress absent special circumstances such as pancreatic disease.

> "*Dietary Fiber* can have variable compositions and therefore it is difficult to link a specific fiber with a particular adverse effect, especially when phytate is also often present. It is concluded that as part of an overall healthy diet, a high intake of *Dietary Fiber* will not produce significant deleterious effects in healthy individuals. Therefore, a Tolerable Upper Intake Level is not set for *Dietary Fiber*" (IOM 2005).

The IOM (2005) panel also examined the need to set a Tolerable Upper Intake Level (UL) for isolated and synthetic fibers (functional fiber), because it is possible to concentrate large amounts of these fibers in foods, beverages, and supplements. The panel suggested that it would be informative to develop projections regarding the potential contribution of functional fiber to daily total fiber intake. Noting that functional fiber, like dietary fiber, is not digested by mammalian enzymes and passes into the colon, the panel determined that any potentially deleterious effects of functional fiber ingestion would be on the interaction with other nutrients in the gastrointestinal tract. The panel summarized its review as follows:

> "While occasional adverse gastrointestinal symptoms are observed when consuming one of the above isolated or synthetic fibers, serious chronic adverse effects have not been observed. Furthermore, due to the bulky nature of fibers, excess consumption is likely to be self-limiting. Therefore, a UL was not set for these individual fibers" (IOM 2005).

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Q9: In the notifier's discussion of human studies, the test articles contained 2'-FL in combination with other indigestible carbohydrates (i.e., FOS in Kajzer et al., 2016, LNnT in Nowak-Wegrzyn et al., 2019). Please provide a brief rationale on intended use of 2'-FL in combination with other indigestible or low-digestible carbohydrates either in infant formula or in conventional foods and the safety and/or tolerability of such combinatorial uses of 2'-FL at the maximum intended use levels in infants, toddlers, and adult subpopulations diet who may be sensitive to indigestible carbohydrates (Grabitske & Slavin, 2009; Livesey, 2001).

Response: DuPont is not a manufacturer of infant formula, enteral feeding products, or conventional foods, and does not at this time contemplate combining 2'FL with other sources of poorly digested carbohydrates. Nevertheless, we recognize the likelihood that manufactures of infant formula, enteral feeding solutions, and conventional foods may choose to use 2'FL in conjunction with other indigestible carbohydrates.

In DuPont's opinion, users of 2'FL and other indigestible carbohydrates must be aware that gastrointestinal tolerance issues may result from excessive concentrations of one or more of these substances. The addition levels of 2'FL presented in DuPont's GRAS notice are representative of appropriate concentrations of human milk oligosaccharides, human-identical milk oligosaccharides, and similar poorly digested carbohydrates.

We expect infant formula manufacturers to use our 2'FL either alone within the level specified in this GRAS notice or in conjunction with other commercially manufactured human milk oligosaccharides within the levels of total oligosaccharides found in human milk, which is inherently well tolerated. Manufacturers might also use our ingredient in conjunction with other indigestible carbohydrates within ranges already established as well tolerated as per clinical trials.

In any event, manufacturers of infant formula, in order to comply with Section 412(d)(1) of the Food, Drug and Cosmetic Act, must—prior to marketing a new formulation—notify FDA and provide a basis for concluding that the formulation, including any content of indigestible carbohydrates, is safe, well tolerated, and able to support normal growth.

For other uses, for which target levels cannot be established based on natural occurrence, appropriate studies of tolerance may be recommended. Here it must be recognized that it is not in a food or enteral formula manufacturer's interest to market a product that causes gastrointestinal intolerance symptoms such as bloating or flatulence or more serious adverse reactions such as diarrhea or constipation. Thus, it is unlikely that a manufacturer planning a total nondigestible carbohydrate level in excess of the levels of 2FL contemplated in this GRAS notice would proceed without first obtaining data to support the safety and tolerability of the target level.

From: jheimbach@va.metrocast.net
To: Hice, Stephanie; jh@jheimbach.com
Cc: "Lim, Angela"; "Jayne Davies"
Subject: RE: GRN 000897 - Questions for Notifier
Date: Monday, April 6, 2020 12:58:27 PM
Attachments: image001.png
April 2020 Revised Table 1.pdf

Dear Dr. Hice—

It turns out that this was merely a transcription error—somehow 8s were misread as 0s, and so ISO 6888-1 was rendered as ISO 6000-1. A corrected version of Table 1 is attached.

We apologize for the error.

Regards—
Jim

James T. Heimbach, Ph.D., F.A.C.N.
JHeimbach LLC
923 Water Street #66
Port Royal VA 22535
USA
Tel: (+1) 804-742-5543
Cell: (+1) 202-320-3063
Email: jh@jheimbach.com

From: Hice, Stephanie <Stephanie.Hice@fda.hhs.gov>
Sent: Thursday, April 2, 2020 3:25 PM
To: jh@jheimbach.com
Cc: 'Lim, Angela' <Angela.Lim@dupont.com>; 'Jayne Davies' <jayne.c.davies@dupont.com>
Subject: RE: GRN 000897 - Questions for Notifier

Dear Dr. Heimbach,

Thank you for your attention to our comments. Upon review of the provided responses, we noted the following:

The notifier states that the method used to detect coagulase positive *Staphylococci* is ISO 6000-1 (revised Table 1). We note that the provided citation does not correspond to an ISO method for the detection of coagulase positive *Staphylococci*. Please provide the appropriate citation for this method.

Thank you, and please let me know if you have any questions.

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

Revised Table 1: Specifications for DuPont's 2'FL

Parameter	Specification	Method	Tested Batches		
			2FL600118	2FL600218	2FL600318
Appearance					
Color	White/off-white	visual	Pass	Pass	Pass
Form	Dry powder	visual	Pass	Pass	Pass
In solution	≤300 ICUMSA[1] units	ICUMSA Method GS 1/3-7	28 ICUMSA	32 ICUMSA	12 ICUMSA
Chemical					
Moisture	≤5%	Karl-Fischer titration	3.6%	4.0%	3.8%
Protein	≤100 µg/g	Bradford Assay	<1.39 µg/g	2.93 µg/g	3.64 µg/g
Total ash	≤0.5%	NMKL 173:2005	0.02%	0.02%	0.01%
Arsenic	≤0.2 mg/kg	EN 15763:2009	<0.015 mg/kg	<0.015 mg/kg	<0.015 mg/kg
Cadmium	≤0.05 mg/kg	EN 15763:2009	<0.001 mg/kg	<0.001 mg/kg	0.008 mg/kg
Lead	≤0.05 mg/kg	EN 15763:2009	<0.007 mg/kg	<0.005 mg/kg	0.006 mg/kg
Mercury	≤0.1 mg/kg	EN 15763:2009	<0.001 mg/kg	<0.001 mg/kg	<0.001 mg/kg
Endotoxins	≤300 EU/g	Ph. Eur. 2.6.14 + Interference study	≤300 EU/g	≤300 EU/g	≤300 EU/g
GMO detection	Negative	PCR (internally validated, EFSA 2018[3])	Negative	Negative	Negative
Carbohydrate					
2FL	>96% (AUC[2])	HILIC[4]	97.8%	98.8%	98.1%
Lactose	<5% (AUC[2])	HILIC[4]	1.7%	0.9%	1.2%
Di-fucosyllactose	<5% (AUC[2])	HILIC[4]	0.6%	0.3%	0.6%
Other CHO (calculated by dfference)	<5% (AUC[2])	HILIC[4]	0.0%	0.0%	0.0%
Microbial					
Standard plate count	<1000 cfu[5]/g	ISO 4833-1	<100 cfu/g	<100 cfu/g	<100 cfu/g
Aerobic contaminants	<5000 cfu/g	ISO 4833-1	<10 cfu/g	<10 cfu/g	<10 cfu/g
Total yeast and mold	<100 cfu/g	ISO 21527	<20 cfu/g	<20 cfu/g	<20 cfu/g
Enterobacteriaceae	Not detected in 10 g	ISO 21528-1	Pass	Pass	Pass
Salmonella spp.	Not detected in 25g (30 individual samples per batch i.e. 30x25g)	ISO 6579-1	Pass	Pass	Pass
Listeria monocytogenes	Not detected in 25 g	ISO 11290-1	Pass	Pass	Pass
Cronobacter sakazakii	Not detected in 10g (30 individual samples per batch i.e.30x10g)	ISO 22964	Pass	Pass	Pass
Coag+ staphylococci	<10 cfu/g (5 x 1 g)	ISO 6888-1	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)
Clostridium perfringens	<10 cfu/g (5 x 1 g)	ISO 7937	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)
Bacillus cereus	<10 cfu/g	ISO 7932	<10 cfu/g	<10 cfu/g	<10 cfu/g
Enterococci	<100 cfu/g	ISO 7899	<50 cfu/g	<50 cfu/g	<50 cfu/g
Clostridia spores	<10 cfu/g (5 x 1 g)	ISO 15213	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)	<10 cfu/g (n = 5)

1. ICUMSA = International Commission for Uniform Methods of Sugar Analysis
2. AUC = area under the curve
3. EFSA 2018. Guidance on the characterization of microorganisms used as feed additives or production organisms; Section 3.2 – evaluation of fermentation products for presence of DNA from the production strain. EFSA Journal. 163:5206.
4. HILIC- Hydrophilic Interaction Liquid Chromatography
5. cfu = colony-forming unit

From: jheimbach@va.metrocast.net
To: Hice, Stephanie
Subject: RE: GRN 000897 - Questions for Notifier
Date: Tuesday, April 21, 2020 3:41:07 PM

Dear Dr. Hice—

Here are the responses to your questions:

1. On page 6 of the notice, the notifier states that the parent strain, *Escherichia coli* strain K12 MG1655 is available from the American Type Culture Collection (ATCC) as ATCC 70926, and that derivation from this parent strain is addressed in GRN 000749, which is incorporated by reference. However, in GRN 000749, *E. coli* strain K12 MG1655 is listed as available from ATCC as ATCC 700926 (page 15 of GRN 000749). Please provide a statement that corrects this reference.

This is a typo with a 0 omitted. The correct ATCC strain designation is indeed 700926 (with two 0's).

2. On page 9 of the notice, the notifier states that "… there is no significant difference between 2FL derived from strain INB000848 and 2FL found in human milk". We note that the strain referenced appears to be incorrect, please provide the correct reference.

This again is a typo with an 8 that should be a 6. The correct strain is INB000846.

Regards,
Jim

James T. Heimbach, Ph.D., F.A.C.N.
JHeimbach LLC
923 Water Street #66
Port Royal VA 22535
USA
Tel: (+1) 804-742-5543
Cell: (+1) 202-320-3063
Email: jh@jheimbach.com