LSRO SOLUTIONS LLC

COMPREHENSIVE GRAS ASSESSMENT

Of the Proposed Uses of

2'-*O*-FUCOSYLLACTOSE

In

Term Infant Formulas, Toddler Formulas, and Foods Targeted to Toddlers

11/17/2017

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II. FOREWORD

Ingredients for use in foods must undergo premarket approval by the U.S. Food and Drug Administration (FDA) as food additives or, alternatively, the ingredients to be incorporated into foods must be determined to be Generally Recognized as Safe (GRAS). In 1997, the FDA altered the GRAS determination process by eliminating the formal GRAS petitioning process and replacing the petitioning process with a notification procedure. While outlining the necessary content to be considered in making a GRAS determination, FDA encouraged that such determinations be provided to FDA in the form of a notification. However, notifying FDA of such determinations is strictly voluntary.

At the request of Danisco USA Inc. (dba DuPont Nutrition and Health) , LSRO Solutions, LLC ("LSRO") has undertaken an independent safety evaluation of 2'-*O*-fucosyllactose (2FL) produced from microbial fermentation to ascertain whether or not the intended use of DuPont's 2FL as an alternative to the existing GRAS uses described herein can be considered to be GRAS based on scientific procedures when used in term infant formula, toddler formula, and food and beverages targeted at toddlers as defined by 21 CFR 170.3(o)(20).

DuPont Nutrition and Health (DuPont) provided background information addressing: the safety/toxicity of 2FL; the intended food uses; and compositional details, specifications, and methods of preparation. DuPont was asked to include adverse reports, as well as those that support conclusions of safety.

Determining how much 2FL can be safely consumed, i.e., the use levels, is critical in the determination of safe exposure levels for 2FL when consumed as a food ingredient, especially in infant formula. The composite safety/toxicity studies, in concert with exposure information, ultimately provide the specific scientific foundation for the GRAS evaluation.

The safety/toxicity studies, consumption/exposure information, and other related documentation were augmented with an independent search of the scientific and regulatory literature conducted by LSRO Solutions, LLC, through June 2017 and summarized in this dossier. A GRAS assessment was developed based on publicly available safety information. Pertinent references are listed in Part 7B.

Tab 1

III. PART 1. SIGNED STATEMENTS AND CERTIFICATIONS

Claim of Exclusion from the Requirement for Premarket Approval [1]

DuPont has concluded that the proposed uses of 2'-*O*-fucosyllactose (2FL) that meet the specifications described herein are GRAS in accordance with Section 201(s) of the Federal Food, Drug, and Cosmetic Act. This conclusion was made in concert with a panel of experts who are qualified by scientific training and experience. The GRAS determination is based on scientific procedures as described in the following sections. The evaluation accurately reflects the conditions of the intended uses of this ingredient in infant formulas, and toddler formulas, foods and beverages targeted to toddlers.

To the best of our knowledge, this determination is a complete, representative, and balanced submission that includes unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the uses of this ingredient in food.

Signed:

(b) (6)

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Date: November 17, 2017

Name and Address of Responsible Party Contact:

Danisco Nutrition and Health
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Wilmington, DE 19803

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[1] Pursuant to 21 CFR 170.35(c)(1); see 81 FR 54960, 17 August 2016: Accessible at https://www.gpo.gov/fdsys/pkg/FR-2016-08-17/pdf/2016-19164.pdf

As the sponsoring party, DuPont accepts responsibility for the conclusion of GRAS status that has been made for DuPont's 2FL as described in the subject claim. Consequently, DuPont's 2FL is exempt from premarket approval requirements for food ingredients.

Common Name and Identity

2'-*O*-fucosyllactose (2FL)

Conditions of Intended Uses in Food

DuPont's 2FL is intended for use as a food ingredient in term infant formulas, toddler formulas, and foods targeted to toddlers to increase the intake of 2FL at the levels listed in Table 1. The intended effect is as a nutrient necessary for the body's nutritional and metabolic processes, serving as a prebiotic for commensal gut bacteria which metabolize prebiotics into short-chain fatty acids used for energy by colonocytes, and to stimulate sodium and water absorption (Engfer et al., 2000; Yates et al., 2007).

Table 1: Proposed Uses for DuPont's 2FL

Food Category	Proposed Food Uses	RACC[a]	Proposed Use Level (g/RACC)	Proposed Maximum Use level [b]
Infant Formulas and Toddler Foods	Infant Formulas	100 mL	0.24	2.4
	Toddler Formulas	100 mL	0.24	2.4
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.84 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2

[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion in the U.S. CFR (21 CFR §101.12)

[b] Proposed maximum use level is presented on g/kg basis for solids, and g/L basis for liquids and forms the basis for the calculation of Estimated Daily Intake presented in Table 9

Basis for GRAS Conclusion

Pursuant to 21 CFR 170.30, conclusions of the GRAS status for 2FL produced by microbial fermentation for use in term infant formula, and specified conventional foods and beverage products intended for use by toddlers, as described herein, has been based on scientific procedures as discussed in the detailed description provided below.

Availability of Information

The data and information that serve as the basis for this GRAS evaluation will be maintained at the offices of DuPont Nutrition and Health, DuPont Experimental Station E320, 200 Powder Mill Road, Wilmington

DE 19803. They will be available for the FDA' s review and copying during customary business hours and a complete copy will be provided to FDA upon request.

Exemptions from Disclosure

This GRAS notice does not contain data and information that is exempt from disclosure under the Freedom of Information Act (FOIA).

However, the signatures and email addresses of the individuals on the various certificates of analysis and statements in Appendices A, D and E have been redacted for reasons of personal privacy; these have no bearing on DuPont's conclusion of GRAS status.

Tab 2

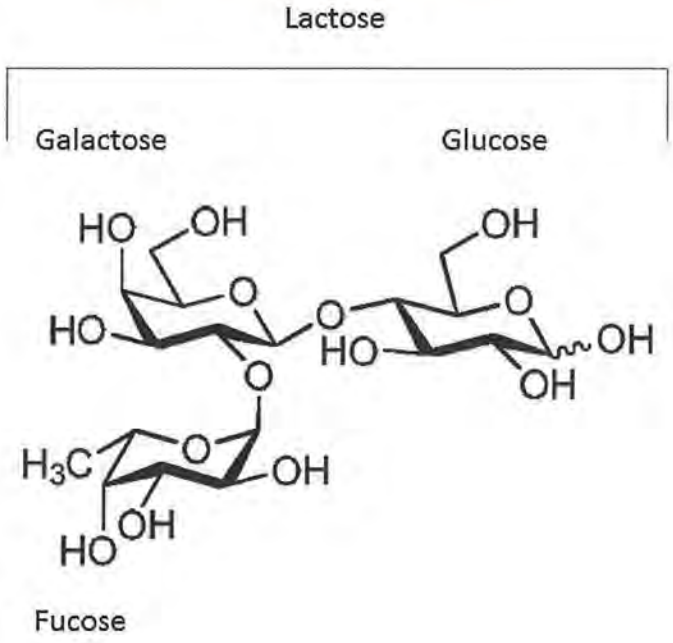
IV. PART 2. IDENTITY, MANUFACTURE, SPECIFICATIONS AND EFFECT

A. Chemical Identity

Common Name:	2'-*O*-fucosyllactose (2FL)
Abbreviation:	2'-FL, 2-FL, 2FL
IUPAC Name:	α-D-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
CAS Number:	41263-94-9
Chemical Formula:	$C_{18}H_{32}O_{15}$
Molecular Weight:	488.44

1. Chemical Structure of 2FL

Figure 1: Chemical Structure of 2FL



2. Chemical and Physical Characteristics

2FL is a trisaccharide, a type of oligosaccharide, consisting of galactose, glucose and fucose (Figure 1). Because the dimeric saccharide lactose is comprised of galactose and glucose, 2FL may also be viewed as consisting of lactose and fucose. The chemical structure of 2FL was determined in 1954 and confirmed using a variety of nuclear magnetic resonance techniques and x-ray crystallography (Castanys-Munoz et al., 2013). Oligosaccharides are polymeric saccharides with a degree of polymerization between 3 to 20; human milk oligosaccharides (HMOs) all contain lactose at their

reducing end. Of the over 400 HMO that have been identified, 2FL is the most abundant (Castanys-Munoz et al., 2013).

DuPont's 2FL produced by fermentation with *Escherichia coli* (*E. coli*) INB3051 is chemically and structurally identical to analytical grade 2FL (IsoSep AB, Tullinge, Sweden) which has been used as the reference standard. This has been confirmed by HPAEC-PAD, HPAEC –MS, ^{1}H-NMR, and ^{13}C NMR. It is also substantially chemically equivalent to the preparations of 2FL that were the subject of three previous GRAS notifications: GRN 650, GRN 571, and GRN 546 (Glycom A/S, 2014, 2016; Jennewein Biotechnologie, 2015) of which the FDA offered no questions upon review.

3. Manufacturing Process

Production host: *E. coli* MG1655 (K12) host with engineered modifications to the chromosome and one gene expressed off a plasmid

Strain name: *E. coli* K12 MG1655 INB3051

Process Description: The DuPont's 2FL production process can be divided up into two main stages: fermentation and post-fermentation processing.

The fermentation stage involves inoculating a small batch of sterilized fermentation media with a seed culture of the production strain. Once the inoculated cells reach an optimal cell concentration, the seeded batch is used to seed increasing volumes of sterilized fermentation media to reach commercial production size batches.

The lactose-sucrose based fermentation media is supplemented with other nutrients such as trace minerals, vitamins and amino acids. Processing aids such as antifoam, pH control agents may also be used in the process. All ingredients/process aids used in the fermentation phases are food or FCC grade and/or are permitted for direct addition to foods as GRAS ingredients and/or food additives.

Fermentation and production chemicals may contain ammonium chloride, ammonium sulfate, potassium phosphates, sodium chloride, citric acid monohydrate, magnesium sulfate, lactose, sucrose, thiamine, zinc chloride, copper chloride, manganese chloride, calcium chloride, iron (II) chloride, glycine, glutamine, methionine, and antifoaming agents.

The fermentation stage is maintained under controlled temperature and pH conditions to optimize growth of the production strain and its expression of 2FL. The resulting fermentate contains various carbohydrates, cell biomass, residual fermentation media, by-products of the fermentation process and other impurities.

The post fermentation processing stage serves to purify and selectively concentrate the 2FL component by removing the cell biomass, residual fermentation media, by-products of the fermentation process and other impurities, to achieve the targeted product purity.

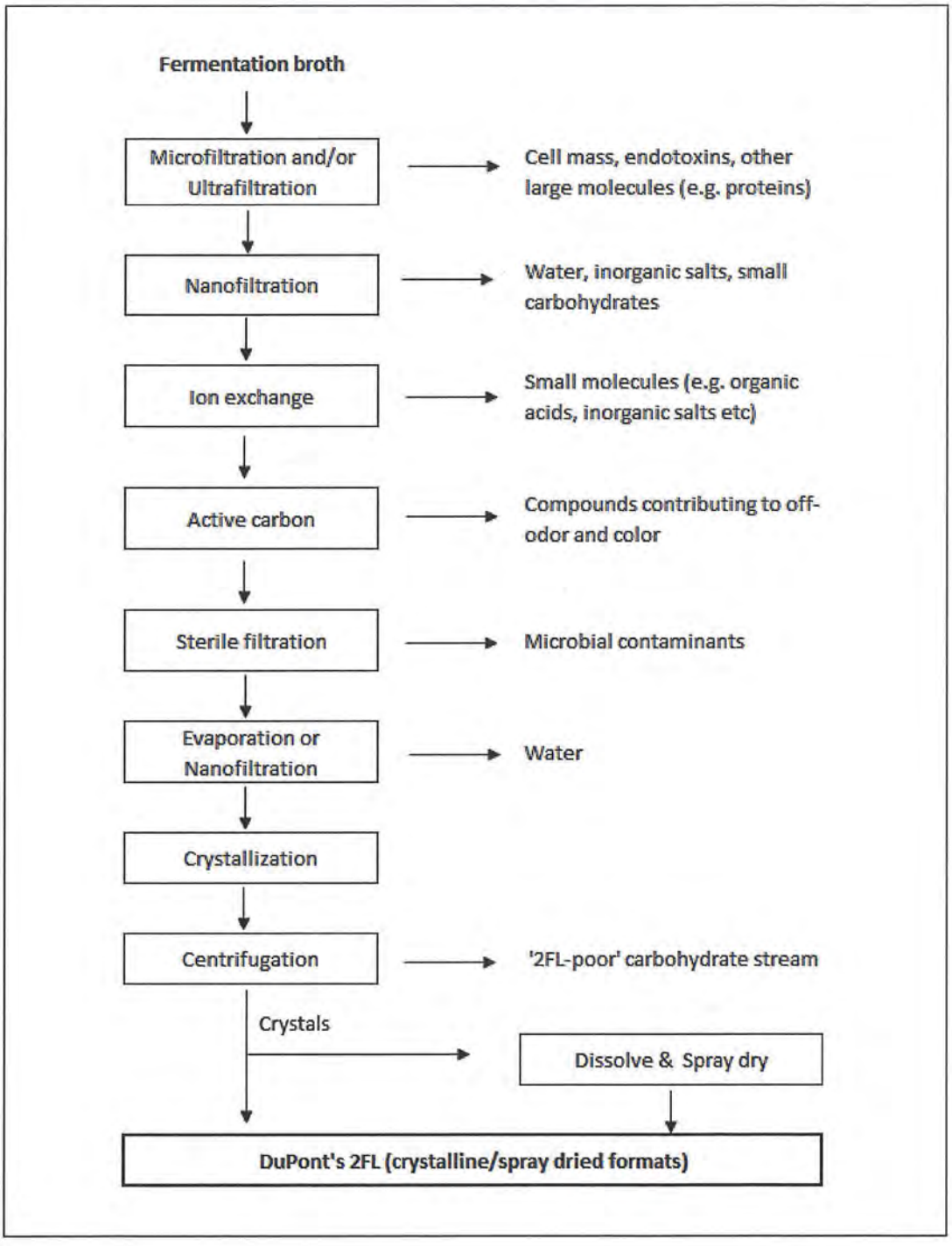
The initial post fermentation processing involves removing the cell biomass, endotoxins and large molecules (e.g. proteins from the fermentate); this is achieved using microfiltration and/or ultrafiltration. The cell-free fermentate is then concentrated using nanofiltration prior to being passed through ion exchange columns and over active carbon where small molecules (e.g. organic acids, inorganic salt) formed/used in the fermentation stage and other compounds that may cause off-color and off-flavor are removed. Potential microbial contaminants are then removed via sterile filtration and the resulting output is concentrated by evaporation/nanofiltration to remove excess water. The concentrated carbohydrate rich fermentate is then crystallized to selectively increase 2FL concentration. Depending upon customer preference, the resulting crystals may be commercialized as is or be dissolved in water then spray dried to yield a spray dried product.

To accommodate production at different manufacturing sites, equivalent technologies/unit operations may be used to accomplish the key purification, concentration and finishing steps outlined.

All processing aids, including but not limited to ion exchange resins, filter aids and regenerating solutions, used in the post fermentation processing stage are approved for use in food processing. DuPont's 2FL product will be manufactured at a site operating in accordance with good manufacturing practice (GMP) and/or Global Food Safety Initiative (GFSI) certifications and meet the requirements of the Food Safety Modernization Act. (Appendix E).

DuPont's 2FL does not contain or consist of GMOs as defined in Regulation (EC) 1829/2003 on genetically modified food and feed, and Regulation (EC) 1830/2003 on the traceability and labelling of genetically modified food and feed products produced from GMOs. Apart from the E. coli K12 production strain, no other genetically engineered ingredients or technology is used in the production of 2FL. The *E. coli* K12 production strain is not present in the finished product. (see Appendix E).

Figure 2: Post fermentation process flow diagram



4. Product Specifications for DuPont's 2FL

Table 2: Product Specifications for DuPont's 2FL

Parameter	Specification	Method
Appearance		
Appearance (color)	White to off-white/ivory	Visual
Appearance (form)	Dry powder	Visual
Appearance in solution	Clear, colorless to slightly yellow	Visual
Chemical Specifications		
Moisture content	≤ 9.0%	Karl Fischer titration
Protein content	≤ 100 µg/g	Nanoquant (modified Bradford)
Total Ash	≤ 0.5%	NMKL 173:2005, mod
Aflatoxin M_1	< 0.025 µg/Kg	EN ISO 14501
Aflatoxin B_1	< 1 µg/Kg	IAC-LC-FLD
Arsenic	≤ 0.2 mg/Kg	EN 15763:2009
Cadmium	≤ 0.05 mg/Kg	EN 15763:2009
Lead	≤ 0.05 mg/Kg	EN 15763:2009
Mercury	≤ 0.1 mg/Kg	EN 15763:2009
Endotoxins	≤ 300 EU/g	Ph. Eur. 2.6.14 + Interference study
GMO detection (rDNA from production strain)	Negative	PCR (internally validated)
Carbohydrate Profile		
2FL	≥ 82% (AUC)	HPAEC-PAD
Lactose	< 8% (AUC)	HPAEC-PAD
Difucosyllactose (DiFL)	< 7% (AUC)	HPAEC-PAD
Other carbohydrates[a] including:	< 6% (AUC)	HPAEC-PAD
3-Fucosyllactose (3FL)		
2-Fucosyl-D-lactulose		
Fucosyl-galactose		
Glucose/Galactose		
Fucose		

Sorbitol/Galactitol		
Mannitol		
Trihexose		
Microbial Specifications		
Standard Plate Count	≤ 1000 cfu/g	ISO 4833
Yeast	≤ 100 cfu/g	NMKL 98
Mold	≤ 100 cfu/g	NMKL 98
Coliform/ Enterobacteriaceae	Not detected in 10 g	ISO 21528-1
Salmonella	Not detected in 100 g	NMKL 71
Cronobacter sakazaki	Not detected in 100 g	ISO/TS 22964
Listeria monocytogenes	Absent in 25 g	BRD 07/04-09/98
Bacillus cereus	≤ 10 cfu/g	NMKL 67-M

[a] Calculated by difference, i.e. 100 – (2FL + lactose + DiFL)

5. Batch Analysis

The results for five production batches of 2FL are tabulated in Table 3 and the certificates of analysis are included in Appendix A. The product is manufactured reproducibly and meets the specifications listed above in Table 2.

Table 3: Analysis of Production Batches of 2FL

Parameter	Specification	Batch Number				
		F13/3	F21	F22	F23	F25
Appearance						
Color	White to off-white/ ivory	Pass	Pass	Pass	Pass	Pass
Form	Dry powder	Pass	Pass	Pass	Pass	Pass
Appearance in solution	Clear, colorless to slightly yellow	Pass	Pass	Pass	Pass	Pass
Chemical						
Water content	≤ 9.0%	5.0	4.6	4.1	3.5	3.2
Protein content	≤ 100 µg/g	≤ 25	≤ 25	≤ 25	≤ 25	≤ 25
Total Ash	≤ 0.5%	< 0.12	< 0.12	< 0.12	< 0.12	< 0.12
Aflatoxin M1	< 0.025 µg/Kg	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Aflatoxin B1	< 1 µg/Kg	< 1	< 1	< 1	< 1	< 1
Arsenic	≤ 0.2 mg/Kg	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

Cadmium	≤ 0.05 mg/Kg	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Lead	≤ 0.05 mg/Kg	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02
Mercury	≤ 0.5 mg/Kg	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Endotoxins	≤ 300 EU/g	<5	<5	10	<5	<5
GMO detection (rDNA from production strain)	Negative	Negative	Negative	Negative	Negative	Negative
Carbohydrate Profile						
2-FL	≥ 82% (AUC)	82.2	86.5	88.1	84.8	83.4
Lactose	< 8% (AUC)	6.5	5.7	2.9	6.8	7.3
DiFL	< 7% (AUC)	6.7	4.7	4.6	4.4	4.2
Other carbohydrates	< 6% (AUC)					
3FL		<0.1	ND	ND	ND	ND
2-Fucosyllactulose		1.10	0.23	0.30	ND	0.95
Fucosylgalactose		0.95	0.51	0.62	0.48	0.77
Glucose/galactose		<0.1	0.42	1.09	1.74	1.14
Fucose		<0.1	ND	ND	ND	0.16
Sorbitol/galactitol		1.02	1.25	1.55	1.12	1.51
Mannitol		<0.1	ND	ND	ND	0.17
Trihexose		1.22	0.67	0.47	0.61	0.48
Total Other Carbohydrates		4.6	3.1	4.0	4.0	5.2
Microbial						
Standard Plate Count	≤ 1000 cfu/g	< 140	< 10	< 10	< 10	< 10
Yeast	≤ 100 cfu/g	< 100	< 10	< 10	< 10	< 10
Mold	≤ 100 cfu/g	< 100	< 10	< 10	< 10	< 10
Coliform/ Enterobacteriaceae	Not detected in 10g	Pass	Pass	Pass	Pass	Pass
Salmonella	Not detected in 100 g	Pass	Pass	Pass	Pass	Pass
Cronobacter sakazaki	Not detected in 100 g	Pass	Pass	Pass	Pass	Pass
Listeria monocytogenes	Not detected in 25 g	Pass	Pass	Pass	Pass	Pass

ND = Not detected, limit of detection is 0.2 g/L

6. Characterization of the Production Organism

The host organism is Escherichia coli K-12 strain MG1655. The taxonomy of the species:

Bacteria
Proteobacteria
Gammaproteobacteria
Enterobacteriales
Enterobacteriaceae
Escherichia
Escherichia coli
Escherichia coli K-12

The host strain, *E. coli* K-12 strain MG1655 is available from both ATCC as 700926 and the Coli Genetic Stock Center as CGSC#7740. *E. coli* strains proliferate via asexual reproduction. This strain is non-recombinant, stable and can easily be maintained as a homogeneous population under the usual laboratory and production conditions. This strain does no produce spores.

E. coli K-12 strain MG1655 is derived from the well-known *E. coli* K-12 strain via classical, non-recombinant genetics and cured of the temperate bacteriophage lambda and F plasmid by means of ultraviolet light and acridine orange, respectively. The genotype of the recipient microorganism is F- lambda- *ilv*G- *rfb*-50 *rph*-1, and the serotype: IRLH48:K- (Blattner et al., 1997). Later additional mutations in commonly used stocks of *E. coli* K-12 strain MG1655 were identified and determined to cause loss of function of the *glpR* and *crl* genes, which are involved in glycerol 3-phosphate and RNA polymerase formation, respectively (Freddolino et al., 2012). The complete genome of this strain has been sequenced (GenBank U00096)[2].

The United States Environmental Protection Agency conducted a risk assessment of *E. coli* K-12 under the Toxic Substances Control Act (U.S. EPA, 1997a, b). This review concluded that "the use of *E. coli* K- 12 under contained conditions in fermentation facilities present low risk" release of this microorganism to the environment through fermentation uses would not pose any significant ecological hazards. based on the following evidence:

1. Wild type *E. coli* is an inhabitant of the human colon.
2. Studies have demonstrated that *E. coli* K-12 is a debilitated strain, defective in at least three cell wall characteristics that are important for colonization. As a result, *E. coli* K-12 is unable to colonize the human intestinal tract under normal conditions. Even in germ free mice, *E. coli* K-12 is a poor colonizer.
3. Evidence indicates indigenous intestinal microorganisms have a large competitive advantage over *E. coli* K-12 strains.
4. *E. coli* K-12 lacks the ability to produce significant quantities of toxins that affect humans. There is no record in the literature of *E. coli* K-12 enterotoxin-induced disease in fermentation workers.
5. *E. coli* K-12 has a history of safe commercial use. Its derivative strains are currently used in a large number of industrial applications, including the product of specialty substances L-aspartic,

inosinic, and adenylic acids which the human body produces, and FDA-approved human drugs such as insulin and somatostatin.

Because *E. coli* K-12 is not considered a human or animal pathogen and is not toxicogenic it falls into Biosafety Level 1 classification and meets the Organization for Economic Cooperation and Development (OECD) GILSP (Good Industrial Large Scale Practice) criteria (Organisation for Economic Co-operation and Development, 1992). The National Institutes of Health considers *E. coli* K-12 to be an organism that does not present a significant risk to health or the environment (National Institutes of Health, 2016). *E. coli* K-12 strain MG1655 has been classified Biosafety Level 1 by the ATCC[3].

Characteristics of the Modified Microorganism

Coding fragments from 4 donor species were synthesized *in vitro* and inserted into the host strain.

First a suitable host strain was produced from parent strain MG1655 by engineering disruptions in genes that interfere with the required metabolic pathway to produce 2FL. The following deletions were made:

lacA (thiogalactoside acetytransferase)
lacZ (beta-D-galactosidase)
glgC (glucose-1-phosphate adenyltransferase)
agp (glucose-1-phosphatase/inositol phosphatase)
pgi (glucose isomerase)
pfkA (6-phosphfructokinase I)
pfkB (6-phosphofructokinase II)
arcA (negative response regulator of genes in aerobic pathways, with sensors ArcB and CpxA)
iclR (regulator in central intermediary metabolism, glyoxylate bypass)
wcaJ (putative UDP-glucose lipid carrier transferase)
lon (enzyme global regulatory function)
adhE (CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrongenase)
ldhA (fermentative NAD-dependent D-lactate dehydrogenase)
fsaB (fructose-6-phosphate aldolase 2)
fsaA (fructose-6-phosphate aldolase)
thyA (thymidiylate synthase)
deletion between *wza* (putative polysaccharide export protein) and *yegH* (putative transport protein)

[2] http://www.ncbi.nlm.nih.gov/nuccore/545778205/
[3] https://www.atcc.org/~/ps/47076.ashx

The following four genes, consisting of codon use adapted coding sequences plus artificial promoters and terminators, were inserted in the genome:

Table 4: Coding DNA Fragment Inserts

Gene	Origin	Length (as bp)	Function	Insertion site
fucT2	*Helicobacter pylori*	900	Fucosyl transferase	Disrupted *ldhA* gene
sucP	*Bifidobacterium adolescentis*	1517	Sucrose phosphorylase	Disrupted *pfkA* gene
frk	*Zymomonas mobilis*	906	Fructokinase	Disrupted *adhE* gene
cscB	*Escherichia coli*	1248	Sucrose transporter	Disrupted *agp* gene

The final strain was constructed by transformation with a plasmid containing the *H. pylori* α-1,2-fucosyl transferase gene (*fucT2*). The host strain contains four genes integrated into the host chromosome, each in one copy and in addition, one multi-copy plasmid containing pBR322 replicon but lacking any conjugation, mobilization or transfer function, and without any antibiotic resistance marker.

The strain proved to be 100% stable after at least 50 generations of fermentation, judged by polymerase chain reaction (PCR) analysis of colonies grown in both selective and non-selective media. The genes integrated into the genome cannot be mobilized or transferred by vector mediated processes such as conjugation. There are no known lytic phages or conjugation plasmids in the host strain and, therefore, transfer can only occur by natural transformation. The integrated genes can be transferred at a frequency normal for chromosomal genes.

The final strain does not contain any trace of the helper plasmid, or from the antibiotic marker used in the construction of the helper plasmid. Some "DNA scars" are left in the genome after constructing gene knock outs or gene insertions. The removal of the helper plasmid is validated by PCR and replica plating on a plate containing the antibiotic for which the marker is present on the helper plasmid.

No specific toxic or allergenic effects are expected from the proteins expressed by the introduced genes. These proteins are not secreted and the cell mass is separated from the product during manufacturing. The absence of these substances is confirmed in the product specification and batch analyses.

A detailed description of the production strain modifications is included in Appendix B.

7. Stability Data

The stability of DuPont's 2FL was studied in accelerated mode (26 wk, 40°C, 75% Relative Humidity). Monitored parameters included carbohydrate content (2FL, DiFL, lactose, other carbohydrates), moisture, and microbiological stability (standard plate count, yeast and mold,

coliform/*Enterobacteriaceae*, *Salmonella*, and *Cronobacter sakazakii*). Three production batches were evaluated. Samples were packed as 5g samples in resealable low-density polyethylene LDPE plastic bags. One 400g sample for each lot was packed for microbiological analyses. Carbohydrate and moisture contents were measured at 0, 1, 4, 8, 13, and 26 wk. Microbiological analyses were performed at 0 and 26 wks.

Stability testing data are presented in Tables 5 and 6. Further details are included in Appendix C. The 2FL content was stable during the 26-week period. Possible minor degradation was reported for one sample, but this was not confirmed by changes in possible carbohydrate degradation products. The moisture content increased during the time period. The product is very hygroscopic and absorbed moisture through the air tight double layered plastic LDPE bags. This did not appear to influence 2FL stability. The discontinuity in moisture values between weeks 4 and 8 was the result of a change in the analytical methodology (from coulometric to volumetric Karl Fischer) and the changes in product properties from powder to paste-like form. The change in analytical methodology was made to ensure better reliability in the data collected. The coulometric Karl Fischer method is applicable for very dry samples (moisture content between 0-10%) and most reliable when moisture is <5%. The volumetric Karl Fischer method is most reliable at moisture levels >5%. The commercial product will be packaged in more moisture impermeable packaging compatible with use as an ingredient in infant formula.

There were no changes in microbiological content over the measurement period. Except for the moisture content, the samples met product specifications throughout the accelerated stability test. A stability test under normal storage conditions (25°C, 60% RH) is ongoing. The carbohydrate profile is unchanged during the 26-week stability study indicating the 2FL is stable even under increased moisture levels.

Table 5: Stability Testing

2FL spray-dried product accelerated stability study (40°C, 75% RH)							
	Specification	**0 wk**	**1 wk**	**4 wk**	**8 wk**	**13 wk**	**26 wk**
Study batch F13/1							
2FL[a]	≥ 82% (AUC)	83.3	83.4	84.7	84.3	83.5	83.6
Lactose	< 8% (AUC)	6.5	6.6	6.2	6.9	6.9	6.8
DiFL + Other carbohydrates	< 13% (AUC)	10.5	10.0	9.1	8.9	9.6	9.6
Moisture[b]	≤ 9.0%	2.2	6.7	7.6	5.5	4.5	3.4
Study batch F13/2a							
2FL[a]	≥ 82% (AUC)	84.7	83.4	84.3	83.6	83.1	83.2
Lactose	< 8% (AUC)	6.7	6.7	6.4	7.3	7.1	7.0
DiFL + Other carbohydrates	< 13% (AUC)	8.7	9.9	9.4	9.2	9.7	9.8
Moisture[b]	≤ 9.0%	2.8	5.8	8.6	8.2	6.3	3.8

Study batch F13/3							
2FL[a]	≥ 82% (AUC)	83.3	83.6	84.0	85.0	82.3	83.6
Lactose	< 8% (AUC)	6.5	6.7	6.5	6.9	8.2	7.1
DiFL + Other carbohydrates	< 13% (AUC)	10.1	9.7	9.5	8.1	9.5	9.3
Moisture[b]	≤ 9.0%	5.0	5.6	9.3	7.7	7.1	5.5

[a] 2FL may contain 2'fucosyllactulose as minor impurity
[b] Moisture analytical methodology changed from coulometric KF to volumetric KF after week 4

Table 6: Microbiological Stability Testing

2FL spray-dried product microbiological accelerated stability study (40°C, 75% RH)			
	Specification	**0 wk**	**26 wk**
Study batch F13/1			
Standard Plate Count	<1000 CFU/g	<10 CFU/g	<10 CFU/g
Yeast and Mold	<100 CFU/g	<100 CFU/g	<10 CFU/g
Coliform/*Enterobacteriaceae*	Not detected in 10g	Not detected in 10g	Not detected in 10g
Salmonella	Not detected in 100g	Not detected in 100 g	Not detected in 100 g
Cronobacter sakazakii	Not detected in 10g	Not detected in 100 g	Not detected in 100 g
Study batch F13/2a			
Standard Plate Count	<1000 CFU/g	<10 CFU/g	<10 CFU/g
Yeast and Mold	<100 CFU/g	<100 CFU/g	<10 CFU/g
Coliform/*Enterobacteriaceae*	Not detected in 10g	Not detected in 10g	Not detected in 10g
Salmonella	Not detected in 100g	Not detected in 100 g	Not detected in 100 g
Cronobacter sakazakii	Not detected in 10g	Not detected in 100 g	Not detected in 100 g
Study batch F13/2			
Standard Plate Count	<1000 CFU/g	<140 CFU/g	Est 70 CFU/g
Yeast and Mold	<100 CFU/g	<100 CFU/g	<10 CFU/g
Coliform/*Enterobacteriaceae*	Not detected in 10g	Not detected in 10g	Not detected in 10g
Salmonella	Not detected in 100g	Not detected in 100 g	Not detected in 100 g
Cronobacter sakazakii	Not detected in 10g	Not detected in 100 g	Not detected in 100 g

The identities of 2FL and related carbohydrates were determined using HPAEC-PAD, mass spectroscopy (MS), ^{1}H-NMR, and ^{13}C-NMR. The 2FL and DiFL components in DuPont's product were compared to pure reference materials isolated from human breast milk (IsoSep AB, Tullinge, Sweden). All major well-resolved signals in the ^{1}H NMR spectra of 2FL (see Appendix D) were identical among materials isolated

from DuPont's 2FL and reference materials, and identical to ^{1}H-NMR spectra reported in the literature (van Leeuwen et al., 2014). The identities of HPAEC peaks for which reference standards were not available but were confirmed by ^{1}H-NMR and heteronuclear single quantum coherence ^{1}H-^{13}C NMR (HSQC), and HPLC-MS analysis.

The similarities among the various commercial 2FL products are compiled in Table 7. While the various products have largely overlapping specification categories, there are differences within these categories. Where the categories are the same, the specifications are comparable. Compared to the product specifications in GRN571 and GRN650, DuPont's 2FL product specifications are slightly lower for 2FL content, and slightly higher for lactose and DiFL. DuPont has not established individual specifications for the minor carbohydrates present in their product, having opted to group these carbohydrates collectively under the heading of 'Other carbohydrates'. Where no specification for DuPont 2FL has been established, the mean analytical value from five nonconsecutive batches is presented. Those values all fall within the product specifications for GRN571 and GRN650. The specifications for proximates and contaminants are all comparable among the various products. Thus, DuPont's 2FL is substantially equivalent to reference materials isolated from human breast milk and other commercial 2FL products that have been concluded to be GRAS by the manufacturers and not objected to by the FDA.

Table 7: Substantial Chemical Equivalence of 2FL from Various Manufacturers

Parameter	DuPont Specification or Mean[a]	Jennewein Specification (GRN 571)	Glycom Specification (GRN 650)
2FL	≥ 82% (Area)	≥ 90% (Area)	≥ 94% (HPLC water-free)
Lactose	≤ 8% (Area)	≤ 5% (Area)	≤ 3.0% w/w%
DiFL	≤ 7% (Area)	≤ 5% (Area)	≤ 1.0% w/w%
Other carbohydrates	<6% (Area)	NS	NS
3-FL	<0.1%[a]	≤ 5% (Area)	NS
2-Fucosyl-D-lactulose	0.8%[a]	NS	≤ 1.0% w/w%
Fucosyl-galactose	0.72%[a]	≤ 3% (Area)	NS
Glucose	1.1%[a]	≤ 3% (Area)	NS
Galactose	1.1%[a]	≤ 3% (Area)	NS
Fucose	0.18%[a]	≤ 3% (Area)	≤ 1.0% w/w%
Total human-identical milk oligosaccharides (2FL, lactose, DiFL, fucose)	95.9%[a]	NS	≥ 96%
Water content	≤ 9.0%	≤ 9.0%	≤ 5%
Protein content	≤ 100 µg/g	≤ 100 µg/g	≤ 100 µg/g
Total Ash	≤ 0.5%	≤ 0.5%	≤ 1.5%
Aflatoxin M_1	< 0.025 µg/kg	≤ 0.025 µg/kg	NS
Aflatoxin B_1	< 1µg / kg	NS	NS

Arsenic	≤ 0.2 mg/kg	≤ 0.2 mg/kg	NS
Cadmium	≤ 0.05 mg/kg	≤ 0.1 mg/kg	NS
Lead	≤ 0.05 mg/kg	≤ 0.02 mg/kg	≤ 0.1 mg/kg
Mercury	≤ 0.1 mg/kg	≤ 0.5 mg/kg	NS
Endotoxins	≤ 300 EU/g	≤ 300 EU/g	*≤ 10 EU/mg*[b]
GMO detection (rDNA from production strain)	Negative	Negative	NS
Appearance (Color)	White to off white/ivory-colored	White to ivory-colored	White to off white
Appearance (Form)	Dry powder	Spray-dried powder	Powder or agglomerates
Appearance in solution	Clear, colorless to slightly yellow	Clear, colorless to slightly yellow	NS

[a] Mean value from five nonconsecutive batches of DuPont 2FL NS= not specified

[b] *According to EFSA doi:10.2903/j.efsa.2015.4184*

8. Biogenic Amines

The potential to produce biogenic amines was evaluated using screening methods for amino acids and biogenic amines[4]. None of the tested batches contained any of the 20 common amino acids or biogenic amines, namely phenyethylamine, spermidine, spermine, histamine, putrescine, cadaverine, tryptamine or tyramine (Appendix A).

9. GMO Status

Other than the *E. coli* K-12 production strain, no other genetically modified ingredients or genetic modification technology was used in the production of the 2FL (Appendix E).

10. Allergens

None of the genes introduced into the production strain were secreted proteins (sucrose phosphorylase, fructokinase, sucrose permease, and fucosyltransferase). Bio-informatic analysis of each of the gene sequences did not reveal a SignalP sequence. SignalP is the sequence for the signal peptide that targets protein excretion into the extracellular space (Nielsen, 2017; Petersen et al., 2011). Moreover, the cell mass is separated from the product during manufacture. The manufacturing facility is free of potential allergens (Appendix E). Batch data and other analyses demonstrate that DuPont's 2FL is consistently below levels of concern of proteins, bacteria or bacterial endotoxins, residual recombinant DNA, and chemical sensitizers including metals. The final product contains lactose.

Tab 3

V. PART 3. DIETARY EXPOSURE

A. Current Dietary Exposure

There is no public information on the current dietary exposures of 2FL to children, teenagers and adults. There is also no public information on the current dietary exposure resulting from addition of 2FL to infant formulas and toddler foods. The estimated All-Person and All-User mean and 90th percentile consumption of the 2FL products described in GRN546, GRN71, and GRN650 are contained in Table 9 in section V.B. In the absence of market data, these represent a very conservative estimate of current usage.

B. Intended Human Food Uses (Estimated Daily Intake)

DuPont's 2FL is intended for use in foods at use levels up to the maximum use levels specified in Table 8. These values are based on GRN650 and adjusted for the relative 2FL content.

Table 8: Intended Human Food Uses of DuPont's 2FL

Food Category	Proposed Food Uses	RACC[a]	Proposed Use Level (g/RACC)	Proposed Maximum Use Level [b]
Infant Formulas and Toddler Foods	Infant Formulas	100 mL	0.24	2.4
	Toddler Formulas	100 mL	0.24	2.4
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.84 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2

[a] RACC = Reference Amounts Customarily Consumed per Eating Occasion in the U.S. CFR (21 CFR §101.12)

[b] Proposed maximum use level is presented on g/kg basis for solid foods and g/L basis for liquid foods & beverages and forms the basis for the calculation of Estimated Daily Intake presented in Table 9

[c] Not applicable

DuPont's 2FL is intended for use in the infant formulas and toddler foods (adjusted for 2FL content) as notified by Glycom in GRN546 and GRN650 and Jennewein in GRN571 (see Table 8). Because the DuPont 2FL product will be replacing products already introduced into the market, the intended use of DuPont 2FL is not expected to noticeably affect the total intake. The EDI of DuPont's 2FL for use in infants and toddlers based on the proposed maximum intended use levels specified in Table 8 is summarized in Tables 9 and 10. The EDI is based on the stratified assessments of dietary intakes of 2FL among U.S. consumers previously reported in GRN546 and GRN650 and adjusted for the 2FL content in Dupont's 2FL. Where applicable, the most recent National Health and Nutrition Examination Surveys (NHANES 2011-2014) have been utilized to estimate the mean and 90th percentile daily intake of 2FL among the U.S. populations.

[4] ISO 13903:2005; EU 152/2009 (F) and Czech J Food Sci, Vol 21

In infants and children, the greatest estimated 90th percentile intake of DuPont's 2FL from all-users and all uses was in infants aged 7 to 12, 8.36 g/day (987.1 mg/kg bw/day). This was followed by infants aged 0 to 6 months, with a level of 5.29 g/day (712 mg/kg bw/day). Use in infant formulas contributed 68.8% to the estimated intakes of 2FL at age 0 to 6 months but only 26.7% in infants aged 7 to 12 months. Use in baby foods for infants and young children made up 64.1% of the total in the older age group.

Table 9: Estimated Daily Intake of DuPont's 2FL

Population Group	Age Group (months)	Consumption (g/person/day)		All-Users Consumption (g/person/day)			
		Mean	90th Percentile	% Users	N	Mean	90th Percentile
From Infant formulas							
Infants[a]	0 – 6	1.51	2.74	74.8	161	2.02	2.91
Infants[a]	7 – 12	1.25	2.49	73.6	128	1.70	2.63
Toddlers[a]	13 – 36	0.01[c]	na	1.1	7	1.08[c]	1.41[c]
From All Proposed Food Uses							
Infants[a]	0 – 6	2.36	5.11	80.5	168	2.93	5.29
Infants[a]	7 – 12	4.63	8.36	100	161	4.63	8.36
Toddlers[b]	13 – 36	1.11	1.96	99.3	561	1.12	1.97

Population Group	Age Group (months)	All-Person Consumption (mg/kg bw/day)		All-Users Consumption (mg/kg bw/day)			
		Mean	90th Percentile	% Users	N	Mean	90th Percentile
From Infant formulas							
Infants[a]	0 – 6	249.0	477.2	74.8	161	332.8	535.6
Infants[a]	7 – 12	138.9	266.5	73.6	128	188.9	295.8
Toddlers[a]	13 – 36	1.0	na	1.1	7	89.3	117.1[c]
From All Proposed Food Uses							
Infants[a]	0 – 6	362.1	681.7	80.5	168	449.7	712.4
Infants[a]	7 – 12	520.1	987.7	100	161	520.2	987.1
Toddlers[b]	13 – 36	84.4	146.0	99.3	558	84.9	146.0

[a] Using 2009-2010 NHANES Data
[b] Using 2011-2012 NHANES Data
[c] Indicates an intake estimate that may not be statistically reliable due to small sample size

C. Estimate of Dietary Exposure of Other Substances

Microbial Endotoxin

Internal specifications for lipopolysaccharides (*i.e.*, endotoxins) originating from the fermentation organism have been established at ≤ 300 EU/g using the *Limulus* amebocyte lysate kinetic chromogenic assay described in the European Pharmacopoeia (Ph.Eur. 2.6.14 + Interference study). This level is consistent with the typical ranges of endotoxin detected in drinking water (Anderson et al., 2002; O'Toole et al., 2008), cow's milk (Gehring et al., 2008), and infant formula powder (Townsend et al., 2007). The analysis of multiple manufacturing batches (Table 2) provides assurance that the 2FL complies with the endotoxin specification.

Production Organism

The production microorganism is efficiently removed in the first step of the downstream processing. Various sequential purification processes are also applied to ensure microbiological purity.

The absence of the microorganisms in the ingredient is demonstrated by microbial testing for *Enterobacteriaceae* and *Escherichia coli* during batch analyses according to internationally-recognized methods (ISO 21528-1:2004). The absence of the production organism in the ingredient is also supported by the analysis of residual bacterial rDNA in batches of the final ingredient (Table 1 and Table 2). Quantitative PCR (qPCR) was utilized to confirm the absence of production organism DNA. Samples of multiple manufacturing batches were lysed, the DNA extracted, and evaluated for rDNA using one primer amplifying a gene on the chromosome (sucrose phosphorylase) and one gene on the plasmid (fucosyl transferase) by qPCR. The limit of detection of this assay was between 0.04885ng/µl and 0.1953 ng/µl for the sucrose phosphorylase primers and between 0.00075 ng/µl and 0.00305 ng/µl for the fucosyl transferase primers. Five manufacturing batches were tested. No rDNA was reported for either the chromosomal or the plasmid primers confirming the absence of the production organism in the final product (Appendix F).

Other HMOs and Carbohydrates

Various monosaccharides, disaccharides, and oligosaccharides have been detected in the final 2FL product. These carbohydrates are natural constituents of human milk or normal components of commercial infant formulas (Castanys-Munoz et al., 2013). Exposure to each of these saccharides as a result of consumption of DuPont's 2FL is insignificant compared to naturally occurring levels at the intended level of use for DuPont's 2FL.

Small but measurable levels of monosaccharides including fucose, glucose, and galactose are present at about 0.1% the level of DuPont's 2FL, levels far below those typically found in human milk. These monosaccharides are normal components of the diet and are readily metabolized.

The lactose content of mature human milk is 67 g/L. Most commercial formulas manufactured in the United States contain lactose as the sole source of carbohydrate at 72 – 74 g/L (Raiten et al., 1998). The

lactose exposure as a result of addition of DuPont's 2FL at its intended use level of 2.4 g/L would be 0.2 g/L. Although in the immature gastrointestinal tract, lactase is virtually absent until at least 34 weeks gestational age, lactose intolerance is quite uncommon before 2 years of age (Heyman, 2006). Lactose malabsorption is not likely to cause significant digestive complications at this low level of lactose and will certainly diminish with increasing lactase expression in the developing gut. Lactose intolerance has not been reported to be an issue with the previously commercialized 2FL products.

3-fucosyllactose (3FL) and difucosyllactose (DiFL) are normal constituents of human milk (McGuire et al., 2017). 3-FL levels range from a low of 0.050 ±0.008 g/L to a high of 0.189 ± 0.012 g/L and DiFL levels range from 0.113 ± 0.017 g/L to 0.298 ± 0.031 nmoles/L. The levels in the U.S. (California) are 0.189 ± 0.023 g/L and 0.237 ± 0.036 g/L for 3FL and DiFL, respectively. By comparison, the level of 2FL in the same population was 3.43 ± 0.28 g/L . 3FL and DiFL levels in DuPont's 2FL were determined using relative area under the curve (AUC) in the HPAEC-PAD chromatogram. At the intended use level of 2.4 g/L of 2FL, the exposure to 3FL would be 0.003 g/L and to DiFL would be 0.19 g/L. These levels are at or below the levels typically found in human milk.

2-fucosyllactulose is the product of isomerization of 2FL during processing. This type of isomerization is pH and temperature dependent. This isomerization is reported to occur during the conversion of D-lactose into D-lactulose during pasteurization of milk, including human donor milk (Beach and Menzies, 1986; de Segura et al., 2012; Schuster-Wolff-Bühring et al., 2010). Infant formulas have been reported to contain D-lactulose at levels of up to 13.7 mmol/L (4.7 g/L) (Beach and Menzies, 1986). D-lactulose has been detected in heat treated human donor milk at low levels in 65% of the samples (de Segura et al., 2012). The exposure to 2-fucosyllactulose via DuPont's 2FL product when used at its intended levels would be 0.03 g/L. Assuming a similar history of safe use as for that in heat treated human donor milk, the exposure to 2-fucosyllactulose is insignificant.

Fucosylgalactose is a naturally occurring breakdown product of 2FL that is formed by cleavage of glucose by enzymatic hydrolysis. Fucosylgalactose is an epitope associated with the H-antigen in humans and has been observed in the urine of humans after oral ingestion of galactose and lactose and after intravenous injection of galactose (Chester et al., 1979). At the intended use level of DuPont's 2FL, the exposure to fucosylgalactose would be 0.03 g/L, at levels readily excreted in the urine after a galactose load and is not expected to pose any adverse risk.

Tab 4

VI. PART 4. SELF-LIMITING LEVELS OF USE

A. Self-limiting

The intended use of DuPont's 2FL is not self-limiting

Tab 5

VII. PART 5. EXPERIENCE BASED ON COMMON USE IN FOODS

A. Other Information on Dietary Exposure and History of Consumption

Background Dietary Intake of 2FL from Human Breast Milk and Cow's Milk Based Infant Formula

After lactose and lipids, the third most abundant component of human breast milk is oligosaccharides. 2FL is the most abundant oligosaccharide in breast milk at 0.06 to 4.65 g/L (Castanys-Munoz et al., 2013; Coppa et al., 2001). The 2FL content of breast milk varies according to geographic location, lactational stage, ethnicity, Lewis-blood group status, and Secretor status (Erney et al., 2000; Gabrielli et al., 2011; Galeotti et al., 2012; McGuire et al., 2017; Thurl et al., 1996). 2FL is produced by the fucosylation of lactose in the lactating mammary gland by the enzyme 1-2 fucosyltransferases (FUT2) (Castanys-Munoz et al., 2013). About 70% of women are Secretors and contain FUT2 in their breast milk, while non-secretors contain a different fucosyl transferase, FUT3. This distinction has a profound effect on 2FL content. Though the 2FL content declines as lactation continues, the volume of breast milk increases throughout the nursing period; as a result, infants born to a Secretor mother may ingest 2-3 gm of 2FL per day (Castanys-Munoz et al., 2013).

Previous studies have reported the 2FL content in mature human breast milk. GRN546 reported a mean content of 2.35 g 2FL/L based on the results of 17 studies of 2FL content in full-term, mature human breast milk. GRN 571 reported approximately 2.6 g 2FL/L in mature milk without providing a basis for how this value was determined. Breast milk values as high as 8.4 g 2FL/L have been reported (Gabrielli et al., 2011; Galeotti et al., 2012; Musumeci et al., 2006). McGuire et al. (2017) reported 2FL levels in breast milk in 11 rural and urban populations. Recalculated to be expressed as mean ± SEM g/L, values in these populations ranged from 0.7 ± 0.1 g 2FL/L in a rural Ghanaian population to 3.44 ± 0.29 in a California (U.S.) population with a mean across all populations equal to 1.93 ± 0.22 g 2FL/L. Chaturvedi et al. (2001) reported a value of 2.43 ± 0.26 g 2FL/L. These compiled means across populations did not consider variations in analytical methodology, small sample size, the lack of statistical sampling, and other complicating factors. However, because they tend to cluster around the same values they provide a useful measure for the purposes of setting a target within the mean 2FL range in breast milk.

2FL is also found in the milk of other mammals such as goat, pigs, chimpanzee, bonobo, and orangutan (Castanys-Munoz et al., 2013; Chaturvedi et al., 2001). The oligosaccharide content of cow's milk is 100 to 1000 fold lower than that in human milk, and less than 1% of the cow's milk oligosaccharides are fucosylated. Cow's milk is the most common milk used in the production of infant formula in the U.S. (USDA, 2009). Thus, the 2FL content of infant formulas is considered negligible (< 2.4 mg/L) (Aldredge et al., 2013; Bode, 2012, 2015; Glycom A/S, 2014; Tao et al., 2010; Urashima et al., 2001).

B. Summary of Regulatory History

1. Regulatory Reviews of the Safety of 2FL

Three previous GRAS notifications were submitted for 2FL preparations intended for use in infant formulas, toddler formulas/foods, and conventional foods and beverages (Glycom A/S, 2014, 2016;

Jennewein Biotechnologie, 2015). These are summarized in Table 10. These notifications all received letters of no objection from the FDA (U.S. FDA, 2014, 2015, 2016).

The Food Safety Authority of Ireland (FSAI) evaluated an application submitted by Glycom A/S for Novel Food Classification in accordance with Article 4 of the novel food Regulation (EC) No. 258/97 (Ireland, 2014). Formula-only proposed intake of 2FL was 417.6 mg/kg bw/d for infants ages 0-6 months, and all users of infant formula and foods specifically designed for young children was 668, 641, and 355 mg/kg bw/day for 4-6-month-olds, 7-12-month olds, and 13-17-month-olds, respectively. In addition, the application proposed use in supplement form at up to 3 g/day as an alternative to food use and which would not be expected to add to the overall daily 2FL intake from food. The FSAI did not identify any safety concerns associated with consumption of 2FL under the proposed food groups and at the intended use levels.

> "[2FL] is a natural constituent of mammalian milk, with human breast milk having the highest levels among animal species, in particular colostrum. The novel ingredient in this application is identical to the corresponding constituent in human breast milk, and is derived from L-fucose and D-lactose through a series of chemical and physical interactions.... The toxicological data provided has not identified any concerns regarding the safety of this ingredient when consumed as intended, even by vulnerable groups such as infants and young children."

The European Food Safety Authority reviewed the safety of 2FL as a novel food ingredient and concluded that 2FL is:

> "safe for infants up to one year of age) when added to infant and follow-on formulas, in combination with LNnT [Lacto-N-neotetraose], at concentrations up to 1.2 g/L of 2'-FL and up to 0.6 g/L of LNnT, at a ratio of 2:1 in the reconstituted formulae; is safe for young children (older than one year of age) when added to follow-on and young-child formulae, at concentrations up to 1.2 g/L of 2-'FL (alone or in combination with LNnT, at concentrations up to 0.6 g/L, at a ratio of 2:1). The Panel also concludes that 2'-FL is safe when added to other foods at the uses and use levels proposed by the applicant." (EFSA Panel on Dietetic Products, 2015).

One of the additional uses considered safe by the panel was for food supplements as defined in Directive 2002/46/EC at a maximum intake of 3.0 g/day[5]. The supplements could be supplied in a solid form, including capsules and tablets and similar forms, liquid form, and syrup-type or chewable form.

Table 10: GRAS Notifications and Self Affirmed GRAS Determinations 2FL

GRN	Year	Substance	Intended Uses	Maximum Use Level	Notification
546	2014	2FL chemically synthesized from benzyl-2-fucosyllactose >95% 2FL	Infant formula (0-12 mos) Toddlers (12-35 mos) Various other uses in conventional foods intended for children and adults	2.4 g/L Ranging from 0.084 - 2.5 gm/L	Glycom (Glycom A/S, 2014) (U.S. FDA, 2014)
571	2015	2FL derived from fermentation with *E. coli* BL21 >90% 2FL	Infant formula (0-12 mos) Toddlers (12-35 mos)	2.0 g/L	Jennewein (Jennewein Biotechnologie, 2015) (U.S. FDA, 2015)
650	2016	2FL derived from fermentation with *E. coli* K-12 >94% 2FL	Term infant formula Toddler formula Other baby foods for infants Other drinks for young children Various other uses in conventional foods intended for children and adults	2.4 g/L 2.4 g/L 12 g/kg 1.2 g/L Ranging from 0.084 - 2.5 g/L	Glycom (Glycom A/S, 2016) (U.S. FDA, 2016)

[5] Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to dietary supplements. OJ L 183, 12.7.2002, p. 51–57.

Tab 6

VIII. PART 6. NARRATIVE

A. Biodisposition of 2FL

Although they are not found exclusively in human milk, 2FLand other soluble milk glycans (SMG) found in human milk are known collectively as human milk oligosaccharides (HMOs).

Castanys-Munoz et al. (2013) summarized the absorption and distribution of soluble milk glycans. They reported that interaction between soluble milk glycans and human cells can occur in the lumen of the digestive tract or systemically following absorption and translocation into the circulation. Prieto (2005) conducted a randomized, controlled, blinded study, in which infants ages 1 to 8 ± 3 days were fed infant formula containing 3 g/L of FOS, non-reducing fructooligosaccharides comprised of three or four associated monosaccharides, or were breastfed for 16 weeks. The study showed that FOS were well-tolerated and some of the sugars entered the plasma and were excreted in the urine. Rudloff et al. (2006) were the first to report that 2FLand 3FL are found in the urine of breastfed infants but not formula fed infants. Based on the 1-2% of some HMOs excreted via infants' urine, the authors' conclude several hundred mg HMO per day circulate in the infants' blood (Rudloff and Kunz, 2012; Rudloff et al., 2012). (Goehring et al.); Rudloff et al. (2012) Goehring et al. (2014) showed that human milk oligosaccharides including 2FL, 3FL, and LNnT are present in the circulation.

Other studies have shown that human milk oligosaccharides are able to enter the small intestine and colon intact and are excreted in the feces (Chaturvedi et al., 2001; Coppa et al., 2001; Sabharwal et al., 1984; Sabharwal et al., 1988; Sabharwal et al., 1991) *In vitro* degradation studies have also shown that 2FLis not digested by human and porcine brush border enzymes.

Vazquez et al. (2017) administered by gavage a single oral dose of 2FL (>95% purity), 6'- sialyllactose (>95% purity), and lacto-N-neotetraose (LNnT;>90% purity) to adult female Sprague Dawley rats (8-10 weeks old) and measured the time course of absorption into the bloodstream and presence in the urine. The doses of 2FLadministered were 0.2, 1, and 5 g/kg bw, the concentrations of 6'-sialyllactose administered were equivalent to 0.2, 1, ad 3.75 g of 2FL/kg bw, and the concentrations of LNnT administered were equivalent to 0.2 and 1 g 2FL/kg bw (n=8 rats/dose group). In another study, these investigators administered to 9 to 11-day-old rat pups (n=5/sex) an oral bolus of 3 mL of 2FLconcentrations of 1, 2.5, 5, or 10 g/L using a soya formula as a vehicle. Blood was collected at 30, 60, 90, 120, 180, and 240 min. In the adult rats, the average concentration of 2FL was 0.0454 ± 0.2 (mean ± SD). The order of abundance and percentage of animals in which the HMO was present were 3'-SL (89.3%), 6'-SL (61.5%), and 2FL (13.1%) prior to any supplementation. After the bolus doses of 2FL, it was present in serum within 30 min, with the maximum serum concentration occurring at 60 min post-administration for the 0.2 g/kg bw dose and 90 to 120 min for the 1, and 5 g/kg bw doses. 2FL remained present in the plasma for the 5-hour duration of the study. Following an oral bolus in adult rats, 2FL excretion was initiated in rats at between 90 and 120 min post administration. After a bolus dose to rat pups, there were no sex-related effects for doses in plasma, absorption curves were dose-dependent, and the amount of circulating 2FL increased over time but did not reach the maximum concentration within the 4 hour block of time. There was a proportional increase in fucose to the concentration of 2FL. which

also reached maximum absorption at 180 min. While there was no effect on serum lactose after 2FL dosing in rat pups; in adult rats, serum lactose increased. The excretion of 2FL was dose-dependent and showed a continuous increase over time.

B. Toxicity Studies on 2FL

Most of the studies that form the basis of this safety assessment have been reviewed in GRN 650, GRN 571, and GRN 546. This evidence is hereby incorporated by reference into this document and was considered in evaluating the GRAS status of DuPont's proposed use of 2FL. Only a synopsis of the pertinent information in these documents is presented below and in Table 11. A more detailed discussion is included in Appendix G. The reader is referred to the previous GRN notifications referenced above for details of these studies.

The genotoxicity of 2FL produced by chemical synthesis as described in GRN546 or produced by fermentation as described in GRN571 and GRN650 was evaluated in the presence and absence of metabolic activation with S9. Treatment with 2FL did not result in a biologically significant increase in the number of revertant colonies compared with the negative control in various Salmonella strains (Coulet et al., 2014; Jennewein Biotechnologie; Verspeek-Rip). In all three instances, the 2FL was reported to be non-mutagenic. The genotoxicity of 2FL produced by chemical synthesis as described in GRN546 was studied in an in vitro mammalian cell gene mutation test in mouse lymphoma cells and mammalian micronucleus test at levels up to 5000 ug/mL. No increases in the frequency of mutations, no evidence of clastogenicity or aneugenicity were reported either short-term or in continuous exposure both in the presence and absence of metabolic activation with S9 (EFSA Panel on Dietetic Products, 2015). In an *in vitro* mammalian cell mutation test, 2FL produced by fermentation did not increase the number of micronucleated peripheral human lymphocytes both in the presence and absence of metabolic activation (Verbaan). In an *in vivo* mammalian micronucleus test, 2FL produced by fermentation as described in GRN571 was reported to be non-clastogenic at levels up to 2000 mg/kg bw/day U.S. FDA (2015). DuPont Nutrition and Health concurs with these conclusions.

Subacute oral toxicity studies were conducted in rats and pigs. In a pilot study, ten female rats (Crl:CD(SD)) were fed a diet containing 10% w/v 2FL produced by fermentation (GRN571, Jennewein) for seven days. No adverse effects on food consumption, body weights, mortality, behavior or appearance (Jennewein Biotechnologie, 2015) were reported. In a 14-day oral tolerability and dose-range finding study in male and female Wistar IGS:Crl:WI (Han) rats , 2FL produced by chemical synthesis (GRN546, Glycom) was administered by gavage to 7-day-old rats at doses of 0, 2000, 5000, or 7500 mg/kg bw/day. Two female rats in the high dose group died and decreased weight gain was reported in the female high dose group. The authors concluded the highest suitable dose for a 90-day study was 5000 mg/kg bw/day (Coulet et al., 2014). In two studies in pigs, 2FL produced by fermentation (Jennewein, GRN571) was administered by gavage to neonatal pigs (n=27 male and n=21 female) at doses of 0, 200, 500 or 2000 mg/kg bw/day for 20 days. The treatment was well tolerated at all doses, there were no treatment-related adverse effects on growth, weight gain, histological and clinical pathology parameters (Hanlon and Thorsrud, 2014). DuPont Nutrition and Health concurs with these conclusions.

Two subchronic oral toxicity studies were conducted in 7-day-old rat pups. 2FL produced by chemical synthesis (GRN546, Glycom) was administered by gavage at doses of 0, 2000, 5000 or 60000 mg/kg bw/day (Coulet et al., 2014). The authors concluded that 2FL was well tolerated at doses of up to 5000 mg/kg bw/day with the exception of transient lower body weight gain and colored feces. However, because there were three unexplained deaths at the 6000 gm/kg bw/day dose, the authors reported a NOAEL of 5000 mg/kg bw/day. EFSA reviewed this evidence as part of a submission for novel food status and based their estimated NOAEL of 2000 mg/kg bw/day on decreased kidney weights and clinical chemistry and hematological effects (EFSA Panel on Dietetic Products, 2015). Glycom reviewed the results of this study in GRN650, pg 30, and noted "that the hematological effects were limited to slight reductions in (<5%) red blood cell count that were not consistent between sexes and were not associated with histopathological or gross pathological correlates. Changes in clinical chemistry parameters were limited to dose responsive reductions in AST levels in both sexes. AST levels were similarly decreased by a comparable magnitude in both males and females of the FOS group (i.e., positive control). In the absence of further clinical chemistry, hematological or histopathological correlates, the reduction in AST levels were not considered adverse". Glycom considers the NOAEL of 5000 mg/kg bw/day to be appropriate. DuPont Nutrition and Health concurs with the conclusions of a NOAEL of 5000 mg/kg bw/day.

In a similarly designed 90-day study, 2FL produced by fermentation (GRN650, Glycom) was administered by gavage to neonatal rat pups at doses of 0, 2000, 4000, and 5000 mg/kg bw/day. There was no test article-related mortality and no test article-related, adverse changes reported in behavior, ophthalmology, urinalysis, hematology, clinical chemistry, organ weights, macroscopic or microscopic histopathology (Penard, 2015). DuPont Nutrition and Health concurs with these conclusions.

In an unpublished study using 4-week-old male and female CD Crl:CD(SD) rats, 2FL produced by fermentation (GRN571, Jennewein) was included in the rat diet at 10% by weight (Jennewein Biotechnologie, 2015). They reported that 2FL was well tolerated with the only notable effects being pale coloration of the feces. There were no deaths reported and no test article-related effects on body weight, body weight gain, food consumption, water consumption, neurological parameters, hematological and blood chemical parameters, urinalysis, ophthalmological observations, organ weight or macroscopic or histopathological findings. The authors determined a NOAEL of 7660 mg/kg bw/day in this single dose study. DuPont Nutrition and Health concurs with these conclusions.

Table 11: Preclinical Testing

In vitro studies				
Test	**System**	**Dose(s) and Duration**	**Results**	**Reference**
Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA 1537 and TA 102 in the absence and presence of metabolic activation	Plate incorporation method: 52, 164, 512, 1600 or 5000 µg 2FL (+/- S9)/plate Pre-incubation method: 492, 878, 1568, 2800 or 5000 µg 2FL (+/- S9)/ plate 2FL purity: 97.6% (GRN650, Glycom)	Non-mutagenic	Coulet et al. (2014)
Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA98, TA100, TA1535, TA 1537 and *E. coli* WP2uvrA in the absence and presence of metabolic activation	Plate incorporation method: 52, 164, 512, 1600 or 5000 µg 2FL (+/- S9)/plate Pre-incubation method: 492, 878, 1568, 2800 or 5000 µg 2FL (+/- S9)/ plate 2FL purity: 97.6% (GRN650, Glycom)	Non-mutagenic	Verspeek-Rip (2015) - Unpublished study in GRN650, page 000042
Bacterial reverse mutation assay	Salmonella typhimurium strains TA98, TA100, TA1535, and TA 1537 and TA 102 in the absence and presence of metabolic activation	Up to 5000 µg/plate Jennewein 2FL (GRN571, Jennewein)	Non-mutagenic	Jennewein Biotechnologie, (2015) - Unpublished study reported in GRN571, page 000040 Study report in Appendix M2, page 000413
Mammalian cell gene mutation test	L5178Y $tk^{+/-}$ mouse lymphoma cells in the presence and absence of metabolic activation	Up to 5000 µg 2FL (+/- S9)/mL 2FL purity: 99% (GRN546, Glycom)	No evidence of mutation in both short-term and continuous experiments	Coulet et al. (2014)

Mammalian micronucleus test (*in vitro*)	Human blood peripheral lymphocytes in the presence and absence of metabolic activation	Up to 2000 µg 2FL/mL 2FL purity: 96.9%	No evidence of clastogenicity or aneugenicity	EFSA Panel on Dietetic Products (2015) - Comment on unpublished Study Report
Mammalian micronucleus test (*in vitro*)	Human blood peripheral lymphocytes	Up to 2000 µg 2FL/mL in the presence and absence of metabolic activation 2FL purity: 97.6% (GRN650, Glycom)	No evidence of clastogenicity or aneugenicity	Verbaan (2015b) - Unpublished study reported in GRN650, page 000042
In vivo study				
Mammalian micronucleus test (*in vivo*)	Rat (Crl:CD (SD)) 5 rats/sex/dose Vehicle control: 0.8% hydroxypropylmethyl cellulose	500, 1000 or 2000 mg Jennewein 2FL/kg bw/day (GRN571, Jennewein)	Non-clastogenic	Jennewein Biotechnologie, (2015) - Unpublished study reported in GRN571, page 000041 Study report in Appendix M1, page 000355
Animal Studies				
Subacute Oral Toxicity Studies				
7-day oral toxicity pilot study	Rat (Crl:CD(SD)) 10 females	Diet including 10% Jennewein 2FL or regular diet without (GRN571, Jennewein)	No differences in food consumption and no mortality, changes in behavior, or changes in appearance	Jennewein Biotechnologie, (2015) - Unpublished study reported in GRN571, page 000041 Study report in Appendix M3, page 000473

14-day oral tolerability and dose-range finding study	Rat (7-day-old rats) Wistar (Crl:WI(Han)), n=5 rats/sex/group	Via gavage 0, 2000, 5000 or 7500 mg 2FL/kg bw/day 2FL purity: 99% Reference control group: 6000 mg FOS*/kg bw/day *Fructooligosaccharide (GRN546, Glycom)	5000 and 7500 mg/kg bw/day 2FL dose groups and FOS group had lower bw on days 0 to 3 than controls. 7500 mg/kg bw/day 2FL dose group, FOS group and to a lesser extent the 5000 mg/kg bw/day 2FL dose group had liquid and/or yellow feces. The highest suitable dose of 2FL for the 90-day study was lower than 7500 mg/kg bw/day.	Coulet et al. (2014)
3-wk subacute oral toxicity study	Pig (Domestic Yorkshire crossbred swine - farm pigs) 27 male & 21 female neonatal piglets; n= 6, 8, 7 and 6 males and 6, 4, 5 and 6 females each the treatment groups respectively Liquid diet via a feeding bowl, filled 6x/day at a dose volume of 500 mL/kg bw/day	0 (control), 200, 500, and 2000 mg/L 2FL treatment groups From 2 days after birth for 3 weeks (GRN571, Jennewein)	Well tolerated at doses of up to 2000 mg/kg and had no adverse effects on growth. Equivalent doses were 291.7 mg/kg bw/day in males and 298.9 mg/kg bw/day in females	Hanlon and Thorsrud (2014)

Subchronic Toxicity Studies				
90-day subchronic oral toxicity study with 4 wk recovery period	Rat (7-day-old Wistar (Crl:WI (Han)) n=10 for 2000 and 4000 mg/kg bw/day dose groups, n=15/sex/in 5000 mg/kg bw/day dose group 5 males & 5 females in separate recovery groups for the control, 2FL and FOS.	Via gavage Control (water), reference control (5000 mg FOS/kg bw/day) and 5000 mg 2FL/kg bw/day, n=15 rats/sex/day dose group; 2000 & 4000 mg 2FL/kg bw/day, n=10 rats/sex/ dose group. 2FL purity: 97.6% (via fermentation) Postnatal day 7 through age 13 weeks (GRN650, Glycom)	No test article-related mortalities, urinalysis parameters, organ weights, macroscopic, histological observations up to 5000 mg/kg bw/day, the highest dose tested.	Penard (2015) - Unpublished study reported in GRN650, page 000037 Study report summary in Appendix A, page 000068
90-day subchronic oral toxicity study with 4 wk recovery period	Rat (7-day-old Wistar (Crl:WI (Han)) n=10 for 2000 and 5000 mg 2FL/kg bw/day dose groups, n=15/sex/in 6000 mg 2FL/kg bw/day dose group) The treatment period was followed by a 28-day recovery period for FOS and high-dose 2FL groups (n=5 rats/sex/dose group).	Via gavage Control (water), reference control (6000 mg FOS/kg bw/day) and 6000 mg 2FL/kg bw/day, n=15 rats/sex/day dose group; 2000 & 4000 mg 2FL/kg bw/day, n=10 rats/sex/ dose group. 2FL purity: 99% Postnatal day 7 through age 13 weeks (GRN546, Glycom)	NOAEL = 5000 mg/kg bw/day reported by Coulet et al. EFSA Panel (2015) concluded NOAEL was 2000 mg/kg bw/day based on decreased relative kidney weight in the 6000 mg/kg bw dose female dose group, two unexplained deaths and hematological clinical effects in the high and mid dose groups. Glycom considered the 5000 mg/kg bw/day NOAEL to be correct; they did not consider the effects at this dose to be adverse. (Glycom GRN650, page 000036).	Coulet et al. (2014) Reviewed by EFSA Panel on Dietetic Products (2015)

90-day study	Rats (Crl:CD(SD)), 4-week-old male and female (n=10/sex/group) and additional satellite groups for blood sampling	Typical rat diet (0% 2FL) or typical rat diet supplemented with 10% Jennewein 2FL purity: 94.1% (GRN571, Jennewein)	NOAEL=7660 mg/kg bw/day for females and 8720 mg/kg bw/day for males	Jennewein Biotechnologie, (2015) - Unpublished study reported in GRN571, page 000042 Study report in Appendix M3, page 000473

Other Animal Studies

A search of the literature returned various animal studies published since 2016. Although none of these studies was designed as safety studies, some have some safety/toxicology endpoints. These are summarized in Appendix G.

Clinical Studies

Human milk contains greater than 400 milk oligosaccharides of which 20 are known as major human milk oligosaccharides (Thurl et al., 2010). Breast milk contains 5–25 g/l of HMO (Gabrielli et al., 2011). The human milk oligosaccharide (HMO) present in the largest amount is 2FLwhich is present at amounts ranging from 0.06 to 4.65 g/L (Erney et al., 2000). The estimated intake of 2FL from human milk is 170–660 mg/kg body weight per day and may range as high asto 1150 mg/kg body weight per day) (EFSA Panel on Dietetic Products, 2015), while the estimated intake of LNnT can be approximated to 20–100mg/kg body weight per day and may be as high as 385 mg/kg body weight per day (EFSA Panel on Dietetic Products, 2015).

Elison et al. (2016) investigated the safety and tolerability of 2FL, lacto-N-neotetraose (LNnT) or a combination of the two in a parallel, double blind randomized, placebo controlled study in 100 healthy individuals (49 women and 51 men). 2FL, LNnT, or 2FL + LNnT (2:1 mass ratio; mix) was given to healthy adults (dissolved in water and consumed with breakfast daily for two weeks at doses 5, 10 or 20 g per day or 2 g of glucose was given as a placebo. The doses consumed each day were selected because they fell within the range of the average daily intake per kg body weight in infants (EFSA Panel on Dietetic Products, 2015). The products used were crystalline powders with a 99·9%. There was no clinically significant change in any parameter measured. None of the study participants dropped out of the study. Forty-four subjects reported fifty-six adverse events, all of which were considered by the authors to be "mild". The adverse events were considered to be a combination of symptoms, for example, flatulence, bloating, and constipation, mostly occurred at the end of the treatment period and were primarily experienced by individuals in the highest dose group. The Gastrointestinal Symptom Rating Scale (GSRS), a self-assessment tool used to assess symptoms related to abdominal pain, indigestion, reflux, diarrhea, and constipation, showed low ratings at the beginning of the study. In addition, the human milk oligosaccharides tested modulated human milk oligosaccharides and specifically increase Bifidobacteria.

The authors reported that 2FL, LNnT, and 2FL + LNnT were safe and well-tolerated and that the combination of 2FL + LNnT was better tolerated than the individual human milk oligosaccharides.

Puccio et al. (2017) conducted a multicenter, randomized, double-blind, controlled study that compared the tolerability and the effects on growth of feeding infants an intact protein, cow's milk– based infant formula (control, n=87) or the same formula with 1.0 g/L 2FL (Glycom) and 0.5 g/L LNnT (test, n=88) from enrollment at age 14 days or younger through age 6 months. The infants were then given a standard follow up formula from age 6 months to one year. There were no differences reported between two groups in weight gains, formula intake, digestive symptoms, behavior patterns, and stool pattern. The infants fed the formula containing 2FL and LNnT had fewer night- time wake-ups at 2 months, fewer parental reports of bronchitis through ages 4 month and 12 months, fewer respiratory tract infection (adverse event cluster) through 12 months, antipyretics use through 4 months, and antibiotics use through 6 and 12 months. There was no significant difference in the percentage of infants who experienced at least one adverse event. The formula that was supplemented with 2FLand LNnT was safe and well-tolerated, and infants fed the formula showed age-appropriate growth.

(EFSA Panel on Dietetic Products, 2015) reviewed the Puccio et al. (2017) study and reported that there was no difference between groups in growth, and that the growth curves were similar to the WHO growth curve. The Panel also reported that the effects of 2FL on stool and microbiota did not raise concerns.

Marriage et al. (2015) conducted a prospective, randomized, controlled, growth and tolerance study in healthy, full term, singleton infants (birth weight=2490 g) who were exclusively fed infant formula (n=189) or human milk (n=65) from enrollment on day 5 of life through to 119 days of life. The formulas used in the study all had a caloric density of 64.3 kcal/dL and contained either galactooligosaccharides (GOS, control formula, 2.4 g/L), 2.2 g/L GOS plus 0.2 g/L of 2FL, or 1.4 g/L GOS plus 1.0 g/L of 2FL. A fourth group of infants was fed human milk. The authors reported that there was no significant difference between feeding groups in body weight, length, or head circumference growth during the study. The feeding groups had comparable average stool consistency, number of stools per day, and percent of feedings associated with spitting up or vomit. 2FLwas found in the plasma and urine of infants fed 2FL, and infants fed 2FLshowed no significant differences in 2FL uptake. There were no significant differences between groups in overall percentage reporting adverse events or serious adverse events. Adverse events included upper respiratory tract symptoms, otitis media, viral infections and oral candidiasis. The authors reported that all of the formulas were well tolerated and there were no safety concerns noted with either formulas containing 2FL.

Goehring et al. (2016) conducted a sub-study that was nested within the randomized, double-blind, controlled growth and tolerance study Marriage et al. (2015) to examine the effect of feeding healthy singleton infants the formulas described above on biomarkers of immune function. Breastfed infants and infants fed formulas containing 2FL were not different but had lower concentrations of plasma inflammatory cytokines than did infants fed the control formula. These markers included interleukin receptor antagonist (IL-1ra), Il-1α, IL-1β, IL-6, and tumor necrosis factor (TNF-α).

Sprenger et al. (2016) conducted an open observatory, single center, longitudinal cohort study that investigated the relationship between representative FUT2 status and HMOs and the relationship between 2FL status and infant growth through age 4 months. Sixteen mother infant pairs comprising 32% of the study population) had low 2FL (mean 27 mg/L; 95% CI of mean 12±42 mg/L) and 34 (68%) had in high 2FL (mean 2170 mg/L; 95% CI of mean 1880±2460 mg/L) at age one month. The distribution was equal for males and females and anthropometric measurements were equal between groups at birth. There were no significant differences between groups for body length, weight, BMI and head circumference through age 4 months.

Smilowitz et al. (2017) investigated the safety and tolerability of bovine milk oligosaccharides (BMO) in humans. In this single-blind, placebo-controlled, crossover trial, 12 healthy infants consumed each supplement for 11 consecutive days in the following order placebo-control (Polycose, a glucose polymer powder (Abbott Nutrition) and the pure lactose-free), low BMO, and high-BMO doses for eleven consecutive days, followed by a 2-wk washout period between each arm of the study. The low BMO doses was 25% of each individual's daily fiber intake and the high BMO was 35 % of each individual's daily fiber intake. Both the low and high BMO doses were well tolerated and no difference was observed between stool consistency from baseline to after consumption of BMO.

C. Summary of Safety Information

1. Summary of Safety Information on 2FL

Three different formulations of 2FL were evaluated in a variety of *in* vivo, preclinical and clinical studies. *In vivo* and *in vitro* genotoxicity studies reported no evidence of mutagenicity or clastogenicity. Subacute oral toxicity studies with 2FL administered by gavage or in the diet reported that 2FL was well-tolerated at doses up to 7500 mg/kg bw/day for seven days.

Subchronic (90 days) toxicity studies in rats with 2FL administered by gavage or in the diet reported NOAELs from 5000 to7660 mg/kg bw/day. Although one analysis of the data estimated a NOAEL of 2000 mg/kg bw/day on the basis of hematological and clinical pathology at the mid dose group, this estimation was disputed by the authors who estimated a NOAEL of 5000 mg/kg bw/day for the same study. The lower estimate was not supported by additional studies. Clinical studies of 2FL-treated infants report that it is well tolerated at levels up to 1 g/L for infants.

Clinical studies in adults report that it is well tolerated at a level of 10 g/day for adults. A dose of 20 g/day was associated only with mild and reversible gastrointestinal symptoms among adults. The key data for our determination of safety are the 90-day subchronic toxicity studies, human clinical studies in adults and infants, and the history of safe use as a component of human breast milk.

2. Acceptable Daily Intake

Based on a NOAEL of 7660 mg/kg bw/day determined by subchronic oral toxicity studies and applying a 100 fold uncertainty safety factor, an ADI of 77 mg/kg bw/day was determined to be safe. Although the intended use in infant and toddler formulas exceeds this level, the Expert Panel considers that the safe history of consumption of 2FL in breast milk justifies the intended use levels.

D. GRAS Criteria

FDA defines "safe" or "safety" as it applies to food ingredients as:

> "reasonable certainty in the minds of competent scientists that the substance is not harmful under the intended conditions of use. It is impossible in the present state of scientific knowledge to establish with complete certainty the absolute harmlessness of the use of any substance."[6]

Amplification is provided in that the determination of safety is to include probable consumption of the substance in question, the cumulative effect of the substance, and appropriate safety factors. It is FDA's operational definition of safety that serves as the framework against which this evaluation is provided.

Furthermore, in discussing GRAS criteria, FDA notes that:

> "General recognition of safety requires common knowledge about the substance throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food."
>
> "General recognition of safety through experience based on common use in food prior to January 1, 1958, shall be based solely on food use of the substance prior to January 1, 1958, and shall ordinarily be based upon generally available data and information."[7]

Practically speaking, the standard for GRAS has become "reasonable certainty of no harm under the intended conditions of use." FDA discusses in more detail what is meant by the requirement of general knowledge and acceptance of pertinent information within the scientific community, i.e., the so-called "common knowledge element," in terms of the two following elements:[8]

- There must be a basis to conclude that there is consensus (but not unanimity) among qualified scientists about the safety of the substance for its intended use, and this is established by relying upon secondary scientific literature such as published review articles, textbooks, or compendia, or by obtaining opinions of expert panels or opinions from authoritative bodies, such as the National Academy of Sciences.

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6 See 21 CFR 170.3(i)

7 See 21 CFR 170.30(a)

8 See 62 FR 18938 (17 April 1997) Accessible at http://www.gpo.gov/fdsys/pkg/FR-1997-04-17/pdf/97-9706.pdf Accessed June 25, 2014.

- Data and information relied upon to establish safety must be generally available; this is most commonly established by utilizing published, peer-reviewed scientific journals; and

The apparent imprecision of the terms "appreciable," "at the time," and "reasonable certainty" demonstrates that the FDA recognizes the impossibility of providing absolute safety in this or any other area (Lu, 1988; Renwick, 1990; Rulis and Levitt, 2009).

E. Common Knowledge Elements for GRAS Conclusions

1. Public Availability of Scientific Information

The key evidence in this determination has been published in a peer review journal. Various other safety assessments, risk assessments, animal and human studies have all been published in peer reviewed journals or made publicly available on government websites.

F. Expert Panel Conclusions

DuPont Nutrition and Health determined that 2'-*O*-fucosyllactose (2FL) is GRAS for use in term infant formula and conventional foods targeted at toddlers on the basis of scientific procedures. This GRAS determination was based on data generally available in the public domain pertaining to the safety of 2FL, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The Expert Panel consisted of the following qualified experts: Joseph Borzelleca, Ph.D. (Virginia Commonwealth University School of Medicine); Edward Carmines, Ph.D. (Independent Consultant); and Roger A. Clemens, Dr.PH, FIFT, CFS, FASN, FACN, CNS, FIAFST, Adjunct Professor, Pharmacology and Pharmaceutical Sciences, University of Southern California. Kara Lewis, Ph.D. (Independent Consultant) and Michael C Falk, Ph.D. (LSRO Solutions LLC), served as technical advisors to the Expert Panel. DuPont Nutrition and Health convened an Expert Panel that independently and collectively critically evaluated all data and information presented herein, and other information deemed appropriate and concluded that the proposed uses of 2FL in term infant formula, and specified conventional foods and beverage products intended for use by toddlers to increase the intake of 2FL are GRAS based on scientific procedures.

The Expert Panel concluded that other qualified experts evaluating the same data and information would concur with their opinion. A summary of the evaluation of data and information reviewed by the Expert Panel and their conclusions is presented in Appendix H.

G. Conclusion of GRAS Status

DuPont Nutrition and Health has concluded that 2FL is GRAS for use in term infant formula and conventional foods and beverage products targeted at toddlers on the basis of scientific procedures.

DuPont Nutrition and Health considers the 90-day subchronic toxicity studies, the human clinical trial experience in adults and children, and the presence of 2FL in breast milk to be the key data in this determination of safety. This GRAS status conclusion is based on data generally available in the public domain pertaining to the safety of 2FL, as discussed herein, and on consensus among a panel of experts (the Expert Panel) who are qualified by scientific training and experience to evaluate the safety of food ingredients. The Expert Panel convened by DuPont Nutrition and Health independently and critically evaluated all data and information presented herein, and concluded that 2FL is GRAS for use in in term infant formula, and specified conventional foods and beverage products intended for use by toddlers to increase the intake of 2FL based on scientific procedures.

Tab 7

IX. PART 7. LIST OF SUPPORTING DATA AND INFORMATION

A. List of Abbreviations

ALP	alkaline phosphatase
ALT	alanine amino transferase
AOCS	American Oil Chemists' Society
AST	aspartate amino transferase
ATSDR	Agency for Toxic Substances and Disease Registry
AV	acid value
Bw	body weight
CaCl2	calcium chloride
CAERS	Adverse Event Reporting System
CAS	Chemical Abstract Service
CAERS	CSAN Adverse Events Reporting System
Ca_2SO_4	calcium sulfate
CFSAN	Center for Food Safety and Nutrition
CGMPs	Current Good Manufacturing Practices
CO2	carbon dioxide
CPA	cyclophosphamide
DMSO	dimethyl sulfoxide
EFSA	European Food Safety Authority
EU	European Union
FCC	Food Chemicals Codex
FDA	Food and Drug Administration
FSNZ	Food Safety New Zealand
FSAI	Food Safety Authority of Ireland
GC	gas chromatography
GI	gastrointestinal
GGT	gamma-glutamyl transferase
GMO	genetically modified organism
GRAS	Generally Recognized As Safe
GRASP	GRAS Petition
GRN	GRAS notification
HAACP	Hazard Analysis and Critical Control Points
Hb	hemoglobin
HCT	hematocrit
HD	high dose
HDL	high-density lipoprotein
IDL	intermediate-density lipoprotein
IOM	Institute of Medicine

ISO	International Standardization Organization
ISSFAL	International Society for the Study of Fatty Acids and Lipids
KCl	potassium chloride
KH_2PO_4	potassium phosphate monobasic KOH potassium hydroxide
K_2SO_4	potassium sulfate
LC-PUFA	long-chain polyunsaturated fatty acid
LD	low dose
LD_{50}	median lethal dose
LDH	lactate dehydrogenase
LDL	low-density lipoprotein
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MD	middle dose
meq	milliequivalents
$MgSO_4 \cdot 7H_2O$	magnesium sulfate
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
NDA	Dietetic Products, Nutrition and Allergies
NDIN	New Dietary Ingredient Notification
NOAEL	No Observed Adverse Effect Level
NR	not reported
OECD	Organization for Economic Cooperation and Development
PCB	polychlorinated biphenyl
PCE	polychromatic erythrocyte
PES	endoperoxide synthase
PGG_2	15-hydroperoxy-9-11-endoperoxide that contains a substituted cyclopentane ring
PGH_2	prostaglandin H2
PGI_2	prostacyclin
ppb	parts per billion
ppm	parts per million
PV	peroxide value
PUFA	polyunsaturated fatty acid
RBC	red blood cell
vLDL	very low density lipoprotetin
WBC	white blood cell

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C. Appendices

Appendix A: Certificates of Analysis

DANISCO
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CERTIFICATE OF ANALYSIS

Probiotics/Cultures & Food Protection

Supplier DuPont Nutrition & Health

Rev. no. 1

Date 21 July 2017

Material: 2'Fucosyllactose

Batch No.: F13/3

Produced in Europe

Test	Result	Specification	Unit
Appearance			
Color	pass	White to ivory	
Form	pass	Spray-dried powder	
Appearance in solution	pass	Clear, colorless to slightly yellow	
Chemical			
Water content	4.95	≤ 9.0	%
Protein content	≤ 25	≤ 100	µg/g
Total Ash	< 0.12	≤ 0.5	%
Aflatoxin M_1	< 0.01	< 0.025	µg/Kg
Aflatoxin B_1	< 1	< 1	µg/Kg
Arsenic	< 0.1	≤ 0.2	mg/Kg
Cadmium	< 0.01	≤ 0.05	mg/Kg
Lead	< 0.02	≤ 0.05	mg/Kg
Mercury	< 0.005	≤ 0.1	mg/Kg
Endotoxins	< 5	≤ 300	EU/g
GMO detection (rDNA from production strain)	negative	Negative	
Carbohydrate Profile			
2-Fucosyllactose (2FL)	82.2	≥ 82	% (Area)
Lactose	6.54	< 8	% (Area)
Difucosyllactose ((DiFL)	6.69	< 7	% (Area)
Other carbohydrates	4.57	≤ 6	% (Area)
- 3-Fucosyllactose (3FL)	- <0.1		% (Area)
- 2-Fucosyllactulose	- 1.1		
- Fucosylgalactose	- 0.95		
- Glucose/galactose	- <0.1		
- Fucose	- <0.1		
- Sorbitol (galactitol)	- 1.02		
- Mannitol	- <0.1		
- Trihexose	- 1.22		

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Test	Result	Specification	Unit
Microbial			
Standard Plate Count	< 140	≤ 1000	cfu/g
Yeast	< 100	≤ 100	cfu/g
Mold	< 100	≤ 100	cfu/g
Coliform/Enterobacteriaceae	absent	absent	in 10 g
Salmonella	absent	absent	in 100 g
Cronobacter sakazaki	absent	absent	in 100 g
Listeria monocytogenes	absent	Absent	in 25 g
Bacillus cereus	< 10	≤ 10	cfu/g

The above product has been analysed by DuPont Nutrition & Health and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

Dagmar Pettke
Quality & Food Safety EMEA
Probiotics / Cultures & Food Protection
DuPont Nutrition & Health

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CERTIFICATE OF ANALYSIS

Probiotics/Cultures & Food Protection

Supplier DuPont Nutrition & Health

Rev. no. 1

Date 21 July 2017

Material: 2'Fucosyllactose

Batch No.: F21

Produced in Europe

Test	Result	Specification	Unit
Appearance			
Color	pass	White to ivory	
Form	pass	Spray-dried powder	
Appearance in solution	pass	Clear, colorless to slightly yellow	
Chemical			
Water content	4.57	≤ 9.0	%
Protein content	≤ 25	≤ 100	µg/g
Total Ash	< 0.12	≤ 0.5	%
Aflatoxin M_1	< 0.01	≤ 0.025	µg/Kg
Aflatoxin B_1	< 1	≤ 1	µg/Kg
Arsenic	< 0.1	≤ 0.2	mg/Kg
Cadmium	< 0.01	≤ 0.05	mg/Kg
Lead	< 0.02	≤ 0.05	mg/Kg
Mercury	< 0.005	≤ 0.1	mg/Kg
Endotoxins	< 5	≤ 300	EU/g
GMO detection (rDNA from production strain)	negative	Negative	
Carbohydrate Profile			
2-Fucosyllactose (2FL)	86.53	≥ 82	% (Area)
Lactose	5.68	< 8	% (Area)
Difucosyllactose ((DiFL)	4.72	< 7	% (Area)
Other carbohydrates	3.07	≤ 6	% (Area)
- 3-Fucosyllactose (3FL)	- nd		% (Area)
- 2-Fucosyllactulose	- 0.23		
- Fucosylgalactose	- 0.51		
- Glucose/galactose	- 0.42		
- Fucose	- nd		
- Sorbitol (galactitol)	- 1.25		
- Mannitol	- nd		
- Trihexose	- 0.67		

nd = not detected

DANISCO
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Test	Result	Specification	Unit
Microbial			
Standard Plate Count	< 10	≤ 1000	cfu/g
Yeast	< 10	≤ 100	cfu/g
Mold	< 10	≤ 100	cfu/g
Coliform/Enterobacteriaceae	absent	absent	in 10 g
Salmonella	absent	absent	in 100 g
Cronobacter sakazaki	absent	absent	in 100 g
Listeria monocytogenes	absent	Absent	in 25 g
Bacillus cereus	< 10	≤ 10	cfu/g

The above product has been analysed by DuPont Nutrition & Health and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

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Probiotics/Cultures & Food Protection

Supplier DuPont Nutrition & Health

Rev. no. 1

Date 21 July 2017

Material: 2' Fucosyllactose

Batch No.: F22

Produced in Europe

Test	Result	Specification	Unit
Appearance			
Color	pass	White to ivory	
Form	pass	Spray-dried powder	
Appearance in solution	pass	Clear, colorless to slightly yellow	
Chemical			
Water content	4.14	≤ 9.0	%
Protein content	≤ 25	≤ 100	µg/g
Total Ash	< 0.12	≤ 0.5	%
Aflatoxin M_1	< 0.01	≤ 0.025	µg/Kg
Aflatoxin B_1	< 1	≤ 1	µg/Kg
Arsenic	< 0.1	≤ 0.2	mg/Kg
Cadmium	< 0.01	≤ 0.05	mg/Kg
Lead	< 0.02	≤ 0.05	mg/Kg
Mercury	< 0.005	≤ 0.1	mg/Kg
Endotoxins	10	≤ 300	EU/g
GMO detection (rDNA from production strain)	negative	Negative	
Carbohydrate Profile			
2-Fucosyllactose (2FL)	88.1	≥ 82	% (Area)
Lactose	2.9	< 8	% (Area)
Difucosyllactose (DiFL)	5.0	< 7	% (Area)
Other carbohydrates	4.0	≤ 6	% (Area)
- 3-Fucosyllactose (3FL)	- nd		% (Area)
- 2-Fucosyl-D-lactulose	- 0.30		
- Fucosylgalactose	- 0.62		
- Glucose/Galactose	- 1.09		
- Fucose	- nd		
- Sorbitol/Galactitol	- 1.55		
- Mannitol	- nd		
- Trihexose	- 0.47		

nd = not detected

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Test	Result	Specification	Unit
Microbial			
Standard Plate Count	< 10	≤ 1000	cfu/g
Yeast	< 10	≤ 100	cfu/g
Mold	< 10	≤ 100	cfu/g
Coliform/Enterobacteriaceae	absent	absent	in 10 g
Salmonella	absent	absent	in 100 g
Cronobacter sakazaki	absent	absent	in 100 g
Listeria monocytogenes	absent	Absent	in 25 g
Bacillus cereus	< 10	≤ 10	cfu/g

The above product has been analysed by DuPont Nutrition & Health and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

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Probiotics/Cultures & Food Protection

Supplier DuPont Nutrition & Health

Rev. no. 1

Date 21 July 2017

Material: 2'Fucosyllactose

Batch No.: F23

Produced in Europe

Test	Result	Specification	Unit
Appearance			
Color	pass	White to ivory	
Form	pass	Spray-dried powder	
Appearance in solution	pass	Clear, colorless to slightly yellow	
Chemical			
Water content	3.47	≤ 9.0	%
Protein content	≤ 25	≤ 100	µg/g
Total Ash	< 0.12	≤ 0.5	%
Aflatoxin M_1	< 0.01	≤ 0.025	µg/Kg
Aflatoxin B_1	< 1	≤ 1	µg/Kg
Arsenic	< 0.1	≤ 0.2	mg/Kg
Cadmium	< 0.01	≤ 0.05	mg/Kg
Lead	< 0.02	≤ 0.05	mg/Kg
Mercury	< 0.005	≤ 0.1	mg/Kg
Endotoxins	< 5	≤ 300	EU/g
GMO detection (rDNA from production strain)	negative	Negative	
Carbohydrate Profile			
2-Fucosyllactose (2FL)	84.8	≥ 82	% (Area)
Lactose	6.82	< 8	% (Area)
Difucosyllactose ((DiFL)	4.44	< 7	% (Area)
Other carbohydrates	3.94	≤ 6	% (Area)
- 3-Fucosyllactose (3FL)	- nd		% (Area)
- 2-Fucosyllactulose	- nd		
- Fucosylgalactose	- 0.48		
- Glucose/galactose	- 1.74		
- Fucose	- nd		
- Sorbitol (galactitol)	- 1.12		
- Mannitol	- nd		
- Trihexose	- 0.61		

nd = not detected

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Test	Result	Specification	Unit
Microbial			
Standard Plate Count	< 10	≤ 1000	cfu/g
Yeast	< 10	≤ 100	cfu/g
Mold	< 10	≤ 100	cfu/g
Coliform/Enterobacteriaceae	absent	absent	in 10 g
Salmonella	absent	absent	in 100 g
Cronobacter sakazaki	absent	absent	in 100 g
Listeria monocytogenes	absent	Absent	in 25 g
Bacillus cereus	< 10	≤ 10	cfu/g

The above product has been analysed by DuPont Nutrition & Health and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

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Rev. no. 1

Date 21 July 2017

Material: 2'Fucosyllactose

Batch No.: F25

Produced in Europe

Test	Result	Specification	Unit
Appearance			
Color	pass	White to ivory	
Form	pass	Spray-dried powder	
Appearance in solution	pass	Clear, colorless to slightly yellow	
Chemical			
Water content	3.22	≤ 9.0	%
Protein content	≤ 25	≤ 100	µg/g
Total Ash	< 0.12	≤ 0.5	%
Aflatoxin M_1	< 0.01	≤ 0.025	µg/Kg
Aflatoxin B_1	< 1	≤ 1	µg/Kg
Arsenic	< 0.1	≤ 0.2	mg/Kg
Cadmium	< 0.01	≤ 0.05	mg/Kg
Lead	< 0.02	≤ 0.05	mg/Kg
Mercury	< 0.005	≤ 0.1	mg/Kg
Endotoxins	< 5	≤ 300	EU/g
GMO detection (rDNA from production strain)	negative	Negative	
Carbohydrate Profile			
2-Fucosyllactose (2FL)	83.42	≥ 82	% (Area)
Lactose	7.26	< 8	% (Area)
Difucosyllactose ((DiFL)	4.15	< 7	% (Area)
Other carbohydrates	5.17	≤ 6	% (Area)
- 3-Fucosyllactose (3FL)	- nd		% (Area)
- 2-Fucosyllactulose	- 0.95		
- Fucosylgalactose	- 0.77		
- Glucose/galactose	- 1.14		
- Fucose	- 0.16		
- Sorbitol (galactitol)	- 1.51		
- Mannitol	- 0.17		
- Trihexose	- 0.48		

nd = not detected

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Test	Result	Specification	Unit
Microbial			
Standard Plate Count	< 10	≤ 1000	cfu/g
Yeast	< 10	≤ 100	cfu/g
Mold	< 10	≤ 100	cfu/g
Coliform/Enterobacteriaceae	absent	absent	in 10 g
Salmonella	absent	absent	in 100 g
Cronobacter sakazaki	absent	absent	in 100 g
Listeria monocytogenes	absent	Absent	in 25 g
Bacillus cereus	< 10	≤ 10	cfu/g

The above product has been analysed by DuPont Nutrition & Health and/or its contract testing laboratory. Analytical results on a representative sample from this batch show that this product meets the above criteria.

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Quality & Food Safety EMEA
Probiotics / Cultures & Food Protection
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eurofins | Scientific Finland

Sample code Nr.	493-2017-00034415	Date	13.7.2017	Page 1 / 2
Analytical Report Nr.	AR-17-FL-014859-01			

Danisco Sweeteners Oy, MT Kantvik Client Code:: FL0000612

Siiri Viikari
Sokeritehtaantie 20
02460 Kantvik
FINLAND

Copy to : Oliver Hasselwander

Technical contact for your orders :			
Sample described as:	F21		
Sample reception date:	03.07.2017	**Analysis starting date:**	03.07.2017
Batch comment:	2 regulatory batches		

			Results
DI004	**DJ**	**Amino acids (acid hydrolysis) Method: ISO 13903:2005; EU 152/2009 (F)**	
(a)		Alanine	<0.015 (LOQ) g/100 g
(a)		Arginine	<0.01 (LOQ) g/100 g
(a)		Aspartic acid	<0.017 (LOQ) g/100 g
(a)		Glutamic acid	<0.021 (LOQ) g/100 g
(a)		Glycine	<0.019 (LOQ) g/100 g
(a)		Histidine	<0.02 (LOQ) g/100 g
(a)		Hydroxyproline	<0.05 (LOQ) g/100 g
(a)		Isoleucine	<0.035 (LOQ) g/100 g
(a)		Leucine	<0.015 (LOQ) g/100 g
(a)		Lysine	<0.014 (LOQ) g/100 g
(a)		Ornithine	<0.05 (LOQ) g/100 g
(a)		Phenylalanine	<0.031 (LOQ) g/100 g
(a)		Proline	<0.02 (LOQ) g/100 g
(a)		Serine	<0.016 (LOQ) g/100 g
(a)		Threonine	<0.006 (LOQ) g/100 g
(a)		Tyrosine	<0.023 (LOQ) g/100 g
(a)		Valine	<0.016 (LOQ) g/100 g
DJ700	**DJ**	**Biogenic Amines (dansyl) Method: Czech J. Food Sci. Vol.21**	
(a)		2-Phenylethylamine	<1 (LOQ) mg/kg
(a)		Cadaverine	<1 (LOQ) mg/kg
(a)		Histamine	<1 (LOQ) mg/kg
(a)		Putrescine	<1 (LOQ) mg/kg
(a)		Spermidine	<1 (LOQ) mg/kg
(a)		Spermine	<1 (LOQ) mg/kg
(a)		Tryptamine	<5 (LOQ) mg/kg
(a)		Tyramine	<1 (LOQ) mg/kg
DJ011	**DJ**	**Cystine, methionine (oxidative) Method: ISO 13903:2005; EU 152/2009 (F)**	
(a)		Cystein +Cystine	<0.006 (LOQ) g/100 g
(a)		Methionine	<0.024 (LOQ) g/100 g

SIGNATURE

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(a) = Accredited analysis

(MU) = Expanded measurement uncertainty (k=2)

DJ - Eurofins Steins Laboratorium (Vejen - Vitamin), DENMARK - DS EN ISO/IEC 17025 DANAK 222

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Sample code Nr.	**493-2017-00034416**	**Date**	**13.7.2017**	**Page 1 / 2**
Analytical Report Nr.	**AR-17-FL-014860-01**			

Danisco Sweeteners Oy, MT Kantvik Client Code:: FL0000612

Siiri Viikari
Sokeritehtaantie 20
02460 Kantvik
FINLAND

Copy to : Oliver Hasselwander [redacted]

Technical contact for your orders :

Sample described as:	F25		
Sample reception date:	03.07.2017	**Analysis starting date:**	03.07.2017
Batch comment:	2 regulatory batches		

			Results
DI004	**DJ**	**Amino acids (acid hydrolysis) Method: ISO 13903:2005; EU 152/2009 (F)**	
(a)	Alanine		<0.015 (LOQ) g/100 g
(a)	Arginine		<0.01 (LOQ) g/100 g
(a)	Aspartic acid		<0.017 (LOQ) g/100 g
(a)	Glutamic acid		<0.021 (LOQ) g/100 g
(a)	Glycine		<0.019 (LOQ) g/100 g
(a)	Histidine		<0.02 (LOQ) g/100 g
(a)	Hydroxyproline		<0.05 (LOQ) g/100 g
(a)	Isoleucine		<0.035 (LOQ) g/100 g
(a)	Leucine		<0.015 (LOQ) g/100 g
(a)	Lysine		<0.014 (LOQ) g/100 g
(a)	Ornithine		<0.05 (LOQ) g/100 g
(a)	Phenylalanine		<0.031 (LOQ) g/100 g
(a)	Proline		<0.02 (LOQ) g/100 g
(a)	Serine		<0.016 (LOQ) g/100 g
(a)	Threonine		<0.006 (LOQ) g/100 g
(a)	Tyrosine		<0.023 (LOQ) g/100 g
(a)	Valine		<0.016 (LOQ) g/100 g
DJ700	**DJ**	**Biogenic Amines (dansyl) Method: Czech J. Food Sci. Vol.21**	
(a)	2-Phenylethylamine		<1 (LOQ) mg/kg
(a)	Cadaverine		<1 (LOQ) mg/kg
(a)	Histamine		<1 (LOQ) mg/kg
(a)	Putrescine		<1 (LOQ) mg/kg
(a)	Spermidine		<1 (LOQ) mg/kg
(a)	Spermine		<1 (LOQ) mg/kg
(a)	Tryptamine		<5 (LOQ) mg/kg
(a)	Tyramine		<1 (LOQ) mg/kg
DJ011	**DJ**	**Cystine, methionine (oxidative) Method: ISO 13903:2005; EU 152/2009 (F)**	
(a)	Cystein +Cystine		<0.006 (LOQ) g/100 g
(a)	Methionine		<0.024 (LOQ) g/100 g

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Sample code Nr.	**493-2017-00034416**	**Date**	**13.7.2017**	**Page 2 / 2**
Analytical Report Nr.	**AR-17-FL-014860-01**			

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(a) = Accredited analysis
(MU) = Expanded measurement uncertainty (k=2)

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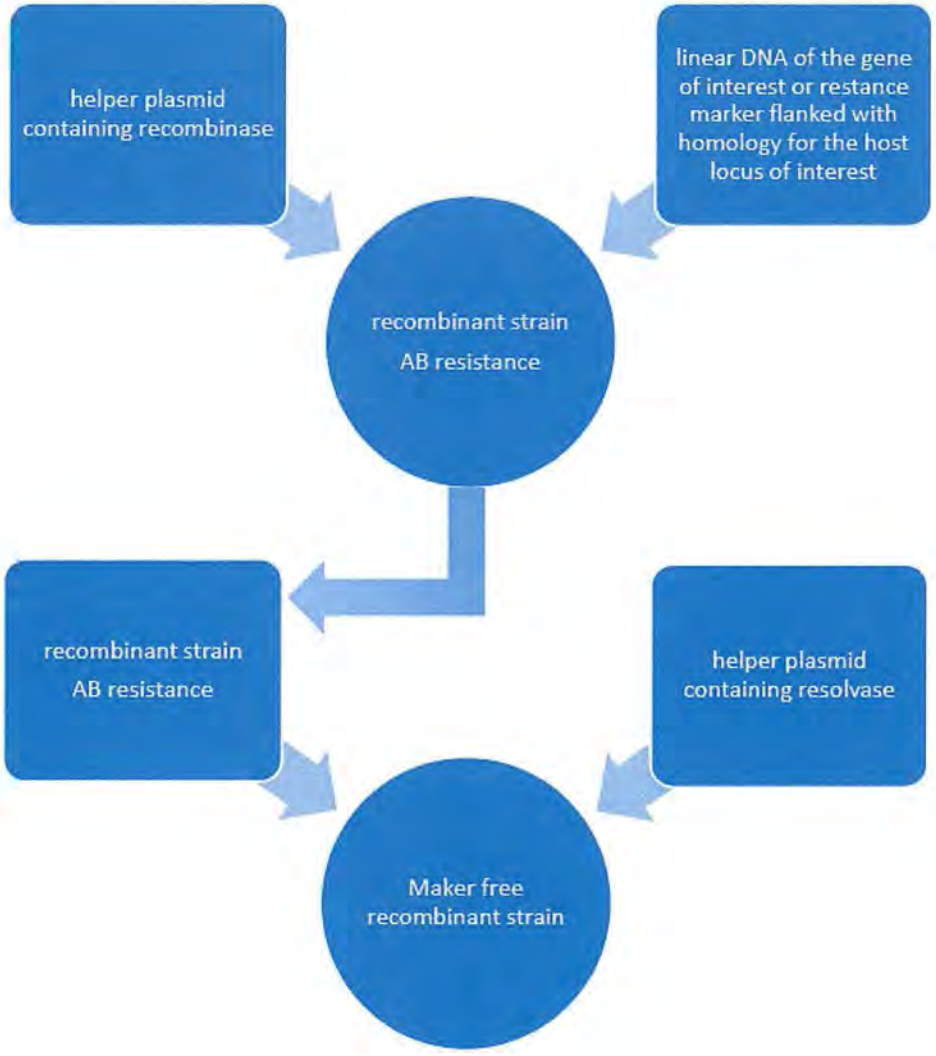
Appendix B: Production Strain Modifications

J. ANNEX 2: DETAILED DESCRIPTION OF THE MODIFICATION.

The production strain was developed by the company Inbiose, Belgium[2], according to the below description. Due to Intellectual Property reasons, not all details are known to DuPont.

1 General workflow of engineering

The general method to introduce modifications into the host genome is show in the scheme below.



The final strain does not contain any trace of the helper plasmid, nor from the antibiotic marker; Typical markers used are: amp, kan, cat, gen, and str. In most instances, some "DNA scars" are left in the genome after constructing gene knock outs or gene insertions (see below).

[2] http://www.inbiose.com/

The removal of the helper plasmid is validated by (1) PCR and (2) replica plating on a plate containing the antibiotic for which the marker is present on the helper plasmid. In the case of the PCR test, no amplification is observed when the plasmid is not present, in the case of the replica plate, no growth is observed for the strains that do not contain the helper plasmid.

In some cases, DNA scars are left behind, although very small and far apart in the chromosome. To date, no chromosomal re-arrangements in the strain have been observed. The host requires an external recombinase to recombine DNA fragments efficiently, the endogenous system requires very large stretches of homology (which are not present in the production host) and is very inefficient. After each modification, each of the previous modifications have been checked by PCR to ensure no other modifications have occurred during the engineering process. Also, no problems with additional modifications or chromosome re-arrangements have been observed. The re-sequencing below further validates this observation.

The methodology used is based on the work of Datsenko & Wanner (2000). The GenBank accession numbers of the plasmids from this method are AY048743.1 (pKD4), AY048742.1 (pKD3), and AY048746.1 (pKD46). These plasmids and a fourth plasmid (pCP20) are shown in Addendum A.

2. Genome loci with modifications

The following loci in the INB3051 strain contain engineered modifications relative to the parent MG1655 (GenBank: U00096.3). In some cases, this resulted in called genetic scars consisting of FRT sites that are left behind due to the used engineering strategy, sometimes including small cloning remnants. In other cases, there are no genetic scars left behind. Nucleotide positions in bold indicate positions relative to MG1655. The codon use in all inserted coding sequences (CDSs) has been adapted. The artificial promoters and terminators used to drive expression of the inserted CDSs are described in De Mey *et. al.* 2007.

>INB3051 locus 360170-362182: contains a clean deletion **361253-361924** = *lacA*, thiogalactoside acetyltransferase + deletion **363180-366377** = *lacZ*, beta-D-galactosidase; + 155bp scar.

>INB3051 locus 3555642-3556341: contains a deletion **3568033-3569328** = *glgC*, glucose-1-phosphate adenylyltransferase; + 84bp scar.

>INB3051 locus 1057841-1060270: contains a deletion **1065506-1066826** = *agp*, glucose-1-phosphatase/inositol phosphatase; + 1859bp insertion containing the *E. coli* anion symport for sucrose (*cscB*) CDS (1135..2382 in GenBank AY314757.1) and a terminator).

>INB3051 locus 4219450-4220212: contains a deletion **4233756-4235410** = *pgi*, glucosephosphate isomerase; + 84bp scar.

>INB3051 locus 4093931-4096403: contains a deletion **4107552-4108514** = *pfkA*, 6-phosphofructokinase I; + 84bp scar; + 1517bp insertion containing *B. adolescentis* ATCC 15703 sucrose phosphorylase (*sucP*) gene (104791..106307 in GenBank AP009256.1).

>INB3051 locus 1796701-1797390: contains a deletion **1806370-1807301** = *pfkB*, 6-phosphofructokinase II; + 84bp scar.

>INB3051 locus 4623795-4624480: contains a deletion **4639590-4640306** = *arcA*, negative response regulator of genes in aerobic pathways, (sensors, ArcB and CpxA); + 84bp scar.

>INB3051 locus 4209371-4209970: contains a clean deletion **4222805-4223667** = *iclR*, regulator; Central intermediary metabolism: Glyoxylate bypass).

>INB3051 locus 2109651-2110533: contains a deletion **2120260-2121453** = *wcaJ*, putative UDP-glucose lipid carrier transferase; + 84bp scar.

>INB3051 locus 454091-454776: contains a deletion **458888-461238** = *lon*, enzyme global regulatory function; + insertion of 79bp *lacP-lacZ* fusion fragment (GenBank AF548059).)

>INB3051 locus 1288311-1290250: contains a deletion **1295446-1298120** = *adhE*, CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase / pyruvate-formate-lyase deactivase; + 1166bp insertion containing *Zymomonas mobilis* subsp. *mobilis* ATCC 10988 fructokinase (*frk*) gene (1633806..1632901 in GenBank CP002850.1).

>INB3051 locus 1431911-1433780: contains a deletion **1441853-1442832** = *ldhA*, fermentative D-lactate dehydrogenase, NAD-dependent; + 1156bp insertion containing *H. pylori* alpha-1,2-fucosyltransferase gene CDS (142..1041 in GenBank AF093828.1).

>INB3051 locus 2125651-2126670: contains a deletion **2137584-2137645** = unknown; between *'wza*, putative polysaccharide export protein' and *'yegH*, putative transport protein'; + 88bp scar.

>INB3051 locus 4126341-4126960: contains a clean deletion **4139078-4140020** = *fsaB*, fructose-6-phosphate aldolase 2).

>INB3051 locus 856601-857200: contains a deletion **863616-864272** = *fsaA*, fructose-6-phosphate aldolase; + 53bp scar.

>INB3051 locus 2952581-2953370: contains a clean deletion **2964361-2965147** = *thyA*, thymidylate synthase).

Via full genome sequencing we also found 18 SNPs in the genome compared to the reference genome. All sequences have now been checked at least 3 times, either PCR combined by sequencing or full genome sequencing.

3 Plasmid based expression

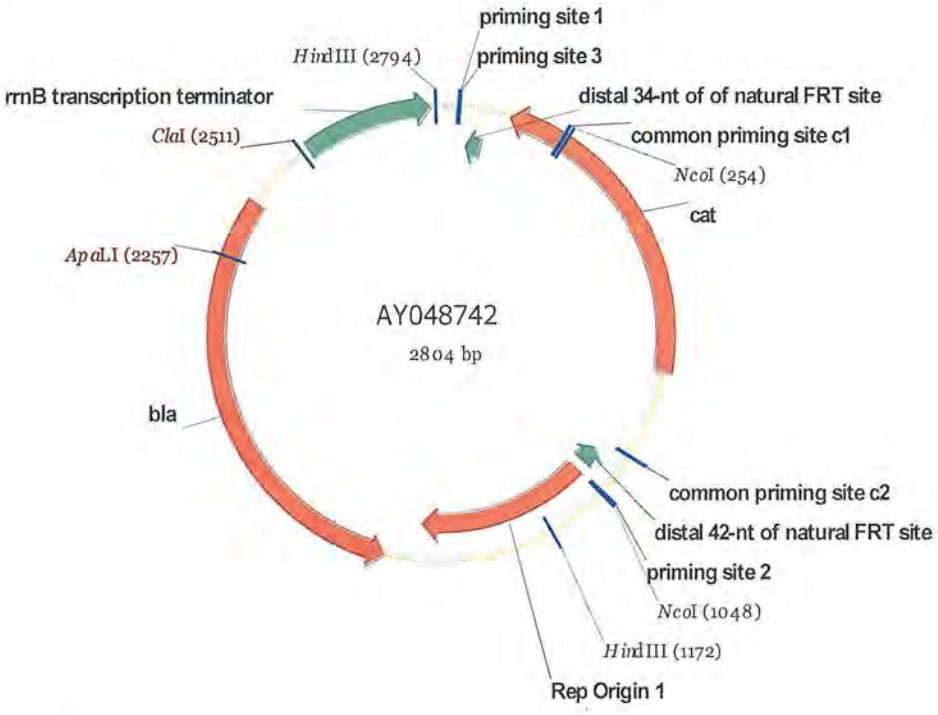
In addition to the chromosomal modifications described above also a plasmid was introduced in the host strain for over expression of the *Helicobacter pylori* fucosyltransferase (*fucT2*) gene, and a *Bifidobacterium bifidum* alpha-fucosidase (*afcA*) gene.

The vector consists of a pCX backbone with pBR322 type *ori* and contains the homologous *E. coli thyA* as a selectable marker, a partial (C-terminal part) *B. bifidum* alpha-fucosidase (*afcA*) gene, and a *H. pylori* fucosyltransferase gene *fucT2*. The *fucT2* gene is controlled by an artificial promoter (P14). The whole vector was synthetized *de novo*. This plasmid pCXP14_*fucT2* is shown in Addendum B.

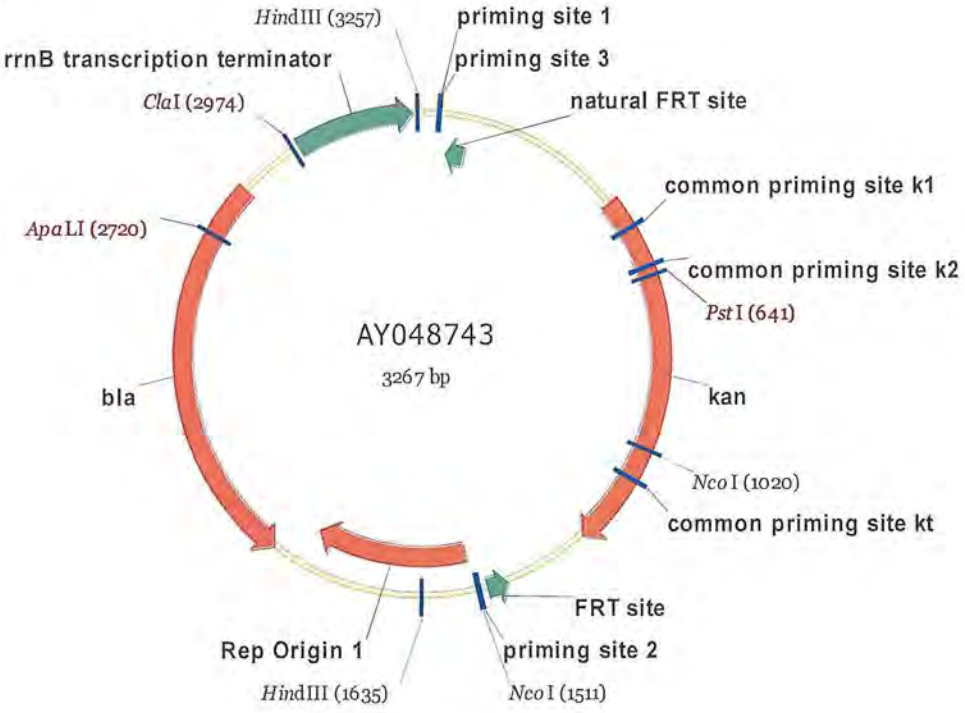
The final strain, INB3051, was deposited in the DuPont Industrial Biosciences Plant Culture Collection as GICC03482

Addendum A

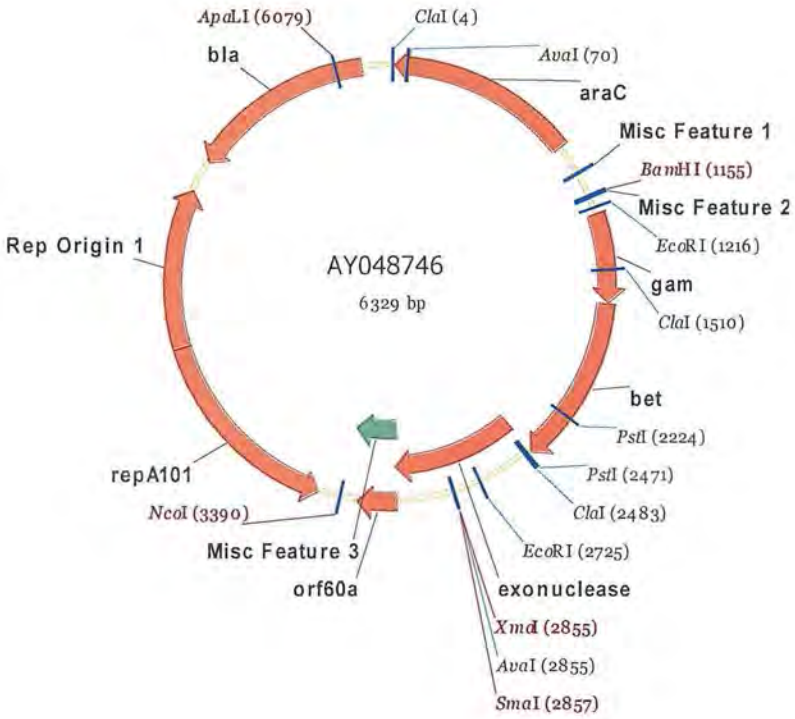
Plasmid pDK3



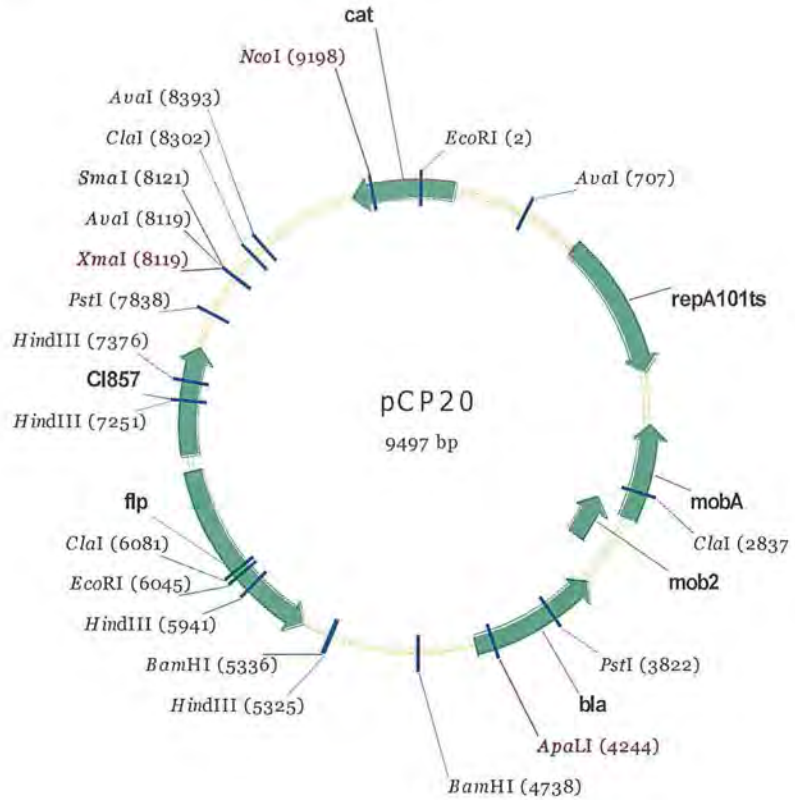
Plasmid pDK4



Plasmid pDK46

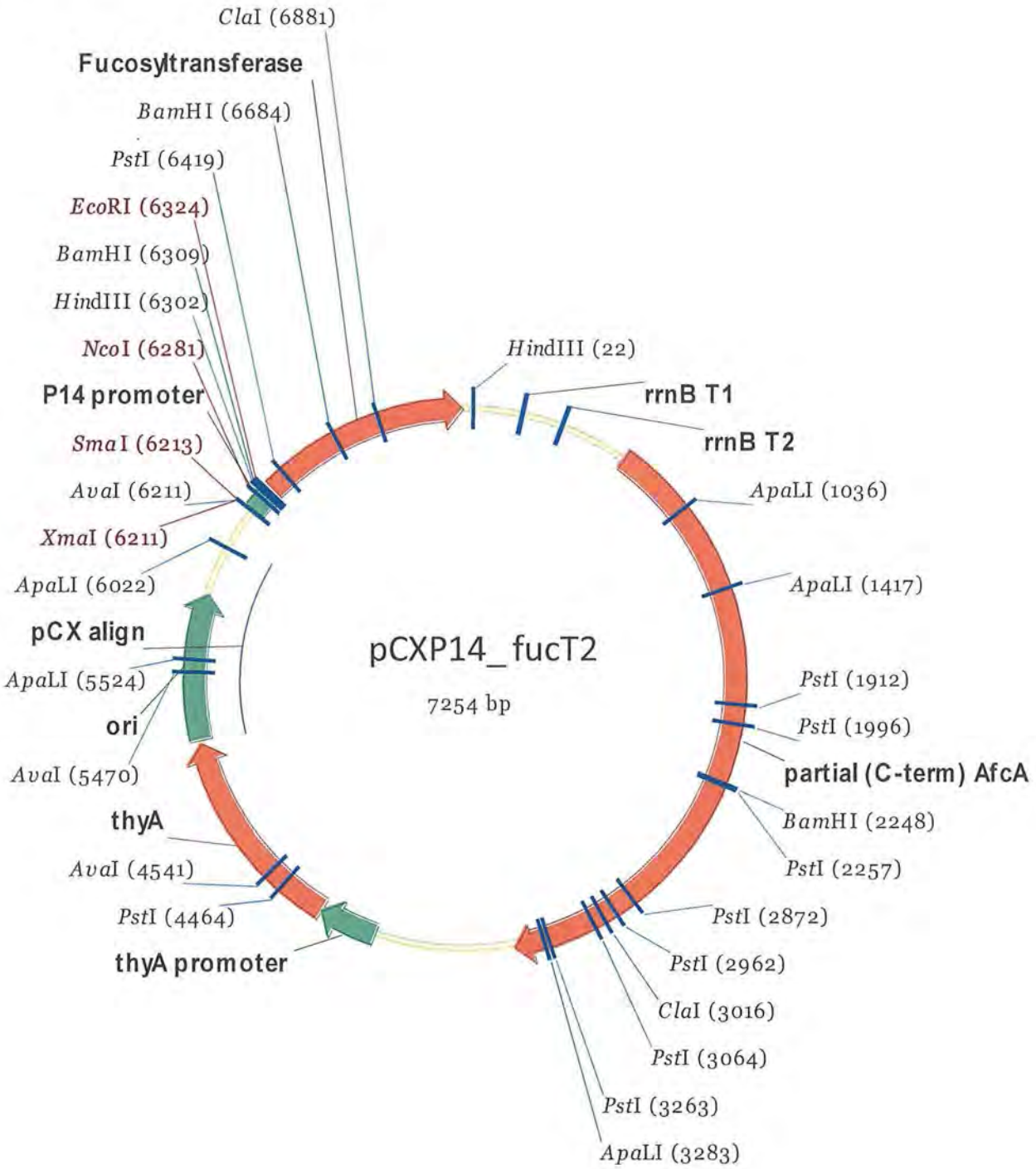


Plasmid pCP20



Addendum B

Plasmid pCXP14_ fucT2



K. ANNEX 3: STABILITY STUDY OF THE PRODUCTION STRAIN

A. Plasmid retention

1 Short description of the experimental setup
The current production host contains an antibiotic marker – free plasmid. The goal of this experiment is to proof that this plasmid is stable over at least 50 generations.

To this end, first the strain is grown in a shake flask and transferred every day to a new shake flask. The number of generations that the strain has gone through is calculated on the number of times the optical density has doubled. Before each transfer to a new shake flask (standard shake flask medium used in the seed train), the strain is diluted in physiological solution to a dilution of 106. This dilution is spread on plate counting agar (selective medium) and enriched plate count agar (non-selective medium, complemented with the marker). From the non-selective medium 10 colonies were picked for PCR on a unique sequence (plasmid sequence).

2 Results of the analysis
To ensure the selective nature of the plate count agar the strain without the plasmid was inoculated on this medium. No growth was observed on these plates without the plasmid, hence the plasmid is essential to grow on the non-selective medium.

Below the results for the production host is shown. For the selective and non-selective medium the colony forming units per mL are given after a given number of generations. The analysis proves that for each sample grown on both media, there is no significant difference in number of colonies[3]. To validate this, 10 colonies from the non-selective medium were tested via PCR and 100% of the colonies still contained the plasmid. Furthermore, no effects were seen on the 2'FL production in shake flask after 50 generations. Hence, the plasmid is not lost after 50 generations.

[3] CFU/ml is affected by the optical density, the optical density sampled was not the same for each generation sampled, hence between the generations sampled there is a lot of variance

Appendix C: Stability Testing

ACCELERATED PRODUCT STORAGE STABILITY STUDY REPORT FOR SPRAY-DRIED 2'-FUCOSYLLACTOSE

Project No. Kantvik: 153.01.02, B60288

1 ABSTRACT

Stability of the spray dried 2'-fucosyllactose (2-FL) was studied in accelerated mode, where samples are stored for 26 weeks in 40 °C (75% RH). In the study, the decomposition of 2-FL was investigated and the microbiological stability was tested. The results showed that 2-FL does not degrade in these conditions significantly and microbiologically the product is stable. It was noticed that chosen packaging was not suitable for this very hygroscopic powder. Moisture absorption was high and physical properties of the powder changed, but it had no influence on the 2-FL content or stability.

2 INTRODUCTION

2'-fucosyllactose (2'-FL) is one oligosaccharide in the group of Human Milk Oligosaccharides (HMO).

2.1 Reason for study

This is a stability study of an existing product and stability needed to be assessed at 40 °C (75%RH) for the regulatory purposes requested by DuPont Regulatory Affairs.

2.2 Objective

To determine the stability of 2'-FL, when stored at 40°C (75%RH) over 26 weeks. The data will be used as a part of the overall stability of the product.

2.3 Study dates

Study start date: 28th November 2016
Study completion date: 29th May 2017

2.4 Testing Locations

Kantvik N&H MT Specialty Carbohydrates stored the samples in study conditions and performed the sampling according to study protocol. Carbohydrate analyses were performed at Kantvik laboratory. Microbiological analyses were performed at Eurofins Scientific Finland Oy.

Address of test facilities:

Kantvik N&H MT Specialty Carbohydrate
Danisco Sweeteners Oy
Sokeritehtaantie 20
02460 Kantvik

FINLAND

2.5 Test material

Three different batches of spray-dried 2'-FL products was provided by Inbiose NV and N&H Brabrand pilot plant. The composition target for 2'-FL product is shown in the table 1. The batches have been all produced by bacterial fermentation by Inbiose NV, batch Ilex 3 F13. One lot (batch F13/1) was purified at Inbiose NV and spray-dried at Institute for Agricultural and Fisheries Research (ILVO). Second lot (F13/2a) was purified at Inbiose NV and spray-dried at N&H Brabrand pilot plant. Third lot (F13/3) was purified at Kantvik N&H MT Specialty Carbohydrates pilot plant and spray-dried at N&H Brabrand pilot plant. All three batches were sent to Kantvik for stability testing.

Table 1. Carbohydrate composition and microbiological target for 2'-fucosyllactose spray-dried product

Parameter	Target value	Method of analyses
Water content	≤ 9%	Coulometric/volumetric KF titration (0525)
CARBOHYDRATE CONTENT		
2'-Fucosyllactose	≥ 83%	HPAEC-PAD (0524)
Lactose	≤ 8% (Area)	HPAEC-PAD (0524)
Difucosyllactose	≤ 9% (Area)	HPAEC-PAD (0524)
Other carbohydrates	≤ 5% (Area)	HPAEC-PAD (0524)
MICROBIOLOGICAL ANALYSES		
Standard Plate Count	≤ 10000 cfu/g	ISO 4833-1
Yeast and Mold	≤ 100 cfu/g	NMKL 98
Coliform / Enterobacteriaceae	absent in 11 g	ISO 21528-1
Salmonella	absent in 100 g	NMKL 71
Cronobacter sakazakii	absent in 100 g	ISO/TS 22964

2.6 Batch details for the study

Table 2. Batch details

Study batch no	Test sample Batch no	2-FL [area%]
1	F13/1	83.0
2	F13/2a	84.7
3	F13/3	83.3
Spare bag	F13/3	83.3

2.7 Responsible personnel

Product manager	:	Steen Lyck, Business Development Leader, PC&FPI BU
Study Scientist	:	Siiri Viikari, Group Manager, Process Analytics, Kantvik N&H MT
Regulatory manager	:	Paul Tenning, Regulatory Manager, Regulatory Affairs EMEA

2.8 Methods of analysis for determining 2'-FL content

HPAEC-PAD method with Dionex PA100 column and NaOH / NaOAc eluent (gradient) originating from Jennewein Biotechnologie GmbH.
Moisture content by Karl Fischer titration.
Details of the methods in **Annex 1**

3. EXPERIMENTAL DESIGN, CRITICAL DATES AND LIST OF DEVIATIONS

1 At t=0, from each study batch a sample was collected and analysed for 2-FL, Difucosyllactose (DiFL) and Lactose contents and for moisture with three parallel samples. Product 2-FL content at time 0 was above target value and moisture content was below the target value and the study was started.

2 All study batch samples were packed as 5g samples (5 pcs) in a resealable LDPE plastic bags, two of them over each other. One 400g sample from each lot was packed for microbiological analyses at time point t=26 weeks.

3 Four 5g bags and one 400g bag from each study batch were placed in the climate cabin and were stored at 40°C (75% RH). 2-FL, DiFL and Lactose contents and moisture was measured at the following time points: 0, 1, 4, 8, 13 and 26 weeks.

4 At each time point one 5g sample bag from each study batch was taken from the climate cabinet.

5 Each 5g sample was analysed with 3 parallel samples for 2-FL, DiFL and Lactose contents and for moisture. Analysis result are expressed as an average of three measurements. The sample solutions were stored for at least until next time point for re-evaluation if needed.

6 Microbiological analyses were performed only at timepoint t=0 and t=26 weeks.

7 Temperature and humidity conditions were recorded with data logger at 12-hour interval. The data logger reports can be found in Annex 2.

3.1 Critical dates

Stability study was started on	28.11.2016
Timepoint t=1 week	5.12.2016
Timepoint t=4 weeks	27.12.2016
Timepoint t=8 weeks	23.1.2017
Timepoint t=13 weeks	27.2.2017

Timepoint t=26 weeks 29.5.2017

3.2 List of deviations

- In the datalogger data in Annex 2 in can be seen that during the stability study the temperature and humidity dropped under limits for short periods for 4 times. These are times, when there was planned power cut in the test facilities. This short drop in the conditions were not considered to have impact on the study.
- In January 2017, there was an equipment error and humidity was not stable for 36 hours. Maintenance was called immediately after failure was noticed and humidity was stabilized.
- Moisture measurement method needed to be changed. Coulometric KF titration is suitable for small moisture levels, but the product absorbed unexpectedly high moisture in the selected packaging and more suitable volumetric KF titration was used from time point t=8 weeks on.

4. RESULTS

At every time point dedicated sample bags were taken from climate cabinet and all samples were analysed for carbohydrate content and for moisture with three parallel measurements. All the measurement results, averages and standard deviations can be found in Annex 3.
A summary of results is in table 3. Carbohydrate contents of stability study samples from both normal and accelerated study at shown timepoints are shown in picture 1.

Table 3. A summary of the measurement results

2-FL Spray-dried product stability, accelerated, 40°C (75% RH)

Study batch/Content		Time Point (weeks)					
		0	1	4	8	13	26
Study batch F13/1							
2 FL*	HPAEC-PAD area under - %	83.3	83.4	84.7	84.3	83.5	83.6
Lactose		6.5	6.6	6.2	6.9	6.9	6.8
DiFL + other carbohydates		10.5	10.0	9.1	8.9	9.6	9.6
Moisture	%	2.2	6.7	7.6	5.5	4.5	3.4
Study batch F13/2a							
2 FL*	HPAEC-PAD area under - %	84.7	83.4	84.3	83.6	83.1	83.2
Lactose		6.7	6.7	6.4	7.3	7.1	7.0
DiFL + other carbohydates		8.7	9.9	9.4	9.2	9.7	9.8
Moisture	%	2.8	5.8	8.6	8.2	6.3	3.8
Study batch F13/3							
2 FL*	HPAEC-PAD area under - %	83.3	83.6	84.0	85.0	82.3	83.6
Lactose		6.5	6.7	6.5	6.9	8.2	7.1
DiFL + other carbohydates		10.1	9.7	9.5	8.1	9.5	9.3
Moisture	%	5.0	5.6	9.3	7.7	7.1	5.5

Note: Moisture measurement changed from coulometric KF to volumetric KF due to limitations of the analytical method

* 2FL result may contain 2 Fucosyl-D-lactulose as a minor impurity

Results show no clear decomposition of 2-FL during the 26-week period. All the variation in the 2-FL results are within the measurement uncertainty of the method (± 2,6%). Only trendline of sample F13/2a shows minor possibility to some degradation, however, the possible degradation products, other carbohydrates, show no increase in content (Picture 2.).

The method for moisture determination was changed from coulometric KF titration to volumetric KF titration after the 4 weeks' time point due to the inherent limitations of the KF titration methods. The coulometric KF titration method is applicable for very dry samples where moisture is between 0-10% and most reliable when moisture is < 5%, whereas the volumetric KF titration method is not reliable at very low moisture levels (<5%). Therefore, as moisture levels increased during storage, it was necessary to change the KF titration methods to ensure better reliability in the data collected.

The carbohydrate results are presented as % of the original content in Table 4. This way it can also be seen that only sample F13/2a shows possible minor degradation during 26 weeks.

Table 4. 2-FL content of stability study samples as % of original content

2-FL Spray-dried product stability, accelerated, 40°C (75% RH)		Time Point (weeks)					
		0	1	4	8	13	26
Study batch F13/1 2 FL	% Original content	100.0	100.5	102.1	101.5	100.6	100.7
Study batch F13/2a 2 FL	% Original content	100.0	98.5	99.5	98.7	98.1	98.2
Study batch F13/3 2 FL	% Original content	100.0	100.4	100.9	102.0	98.8	100.4

Product is very hygroscopic and absorbs moisture through air tight double layered plastic LDPE bags. This influences the product properties but show no influence on 2-FLstability. The decrease in moisture towards the end of the timepoints is due to change in the measurement method from coulometric KF to volumetric KF and the change in product properties from powder form to more paste like form. The commercial product should be packed in more moisture impermeable packaging.

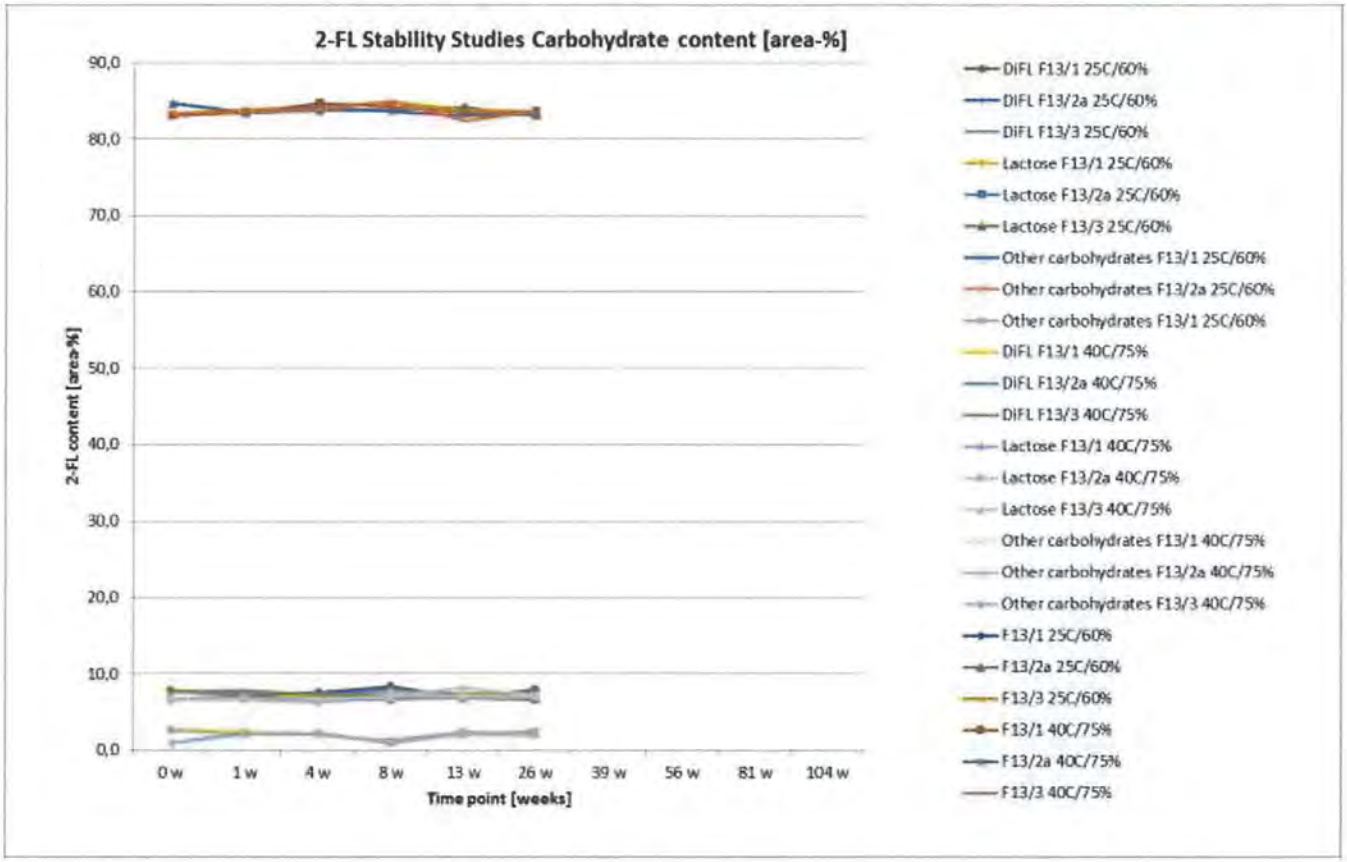
Microbiological stability was tested only at timepoints t=0 and t=26 weeks. The results are shown in Table 5 and show no deterioration.

Table 5. Microbiological results at timepoints t=0 and t=26 weeks

2-FL Spray-dried product microbial stability, accelerated, 40°C (75% RH)

Study batch/Content	Target	Time Point (weeks)	
		0	26
Study batch F13/1			
Standard Plate Count	≤ 10000 cfu/g	< 10 cfu/g	< 10 cfu/g
Yeast & Mold	≤ 100 cfu/g	< 100 cfu/g	< 10 cfu/g
Coliform/Enterobacteriaceae	Absent in 10g	Not detected	Not detected
Salmonella	Absent in 100g	Not detected	Not detected
Cronobacter sakazakii	Absent in 100g	Not detected	Not detected
Study batch F13/2a			
Standard Plate Count	≤ 10000 cfu/g	< 10 cfu/g	< 10 cfu/g
Yeast & Mold	≤ 100 cfu/g	< 100 cfu/g	< 10 cfu/g
Coliform/Enterobacteriaceae	Absent in 10g	Not detected	Not detected
Salmonella	Absent in 100g	Not detected	Not detected
Cronobacter sakazakii	Absent in 100g	Not detected	Not detected
Study batch F13/3			
Standard Plate Count	≤ 10000 cfu/g	140 cfu/g	Est 70 cfu/g
Yeast & Mold	≤ 100 cfu/g	< 100 cfu/g	< 10 cfu/g
Coliform/Enterobacteriaceae	Absent in 10g	Not detected	Not detected
Salmonella	Absent in 100g	Not detected	Not detected
Cronobacter sakazakii	Absent in 100g	Not detected	Not detected

This was an accelerated stability study and at the same time a 104-week stability study at normal conditions (+25 °C / 60 % RH) was started. The results from this accelerated study together with 104-week stability study in normal conditions after 26 weeks don't give any indication that 2-FL product would be instable. In picture 3 there is shown all the carbohydrate contents of the samples from both normal and accelerated stability study until timepoint t=26 weeks.



Picture 1. Carbohydrate contents of stability study samples from both normal and accelerated study at shown timepoints.

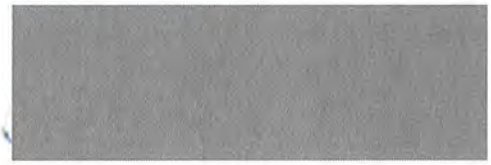
5. STUDY RECORD ACCEPTANCE

Regulatory responsible person

Signature: 

Date: 12/6/2017

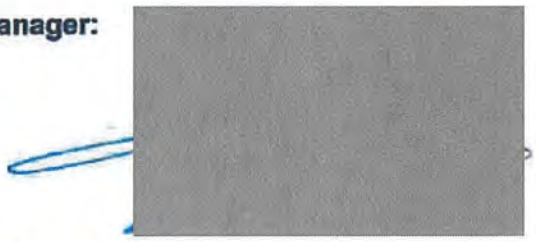
Study scientist from N&H R&D:

Signature: 

Date: 9.6.2017

Acceptance of study on behalf of N&H Management

Product Manager:

Signature: 

Date: 9/6/2017

ANNEXE

ANNEX 1

ANNEX 2

ANNEX 3

Toimintakäsikirja

Monitoring 2-'fucosyllactose and related compounds in the fermentation or in the purification process using high performance anion exchange chromatography with electrochemical detector (HPAEC-PAD) with Dionex PA100 column

1 Applicability

The method is applicable to the determination of 2'-fucosyllactose in process samples during the fermentation reaction and purification steps.

2 Safety Sodium Hydroxide, 50% solution is very corrosive substance. Wear suitable gloves and eye/face protection.

Nitrogen gas is used while working with Dionex ICS 3000 equipment, get to know the gas alarm system in the laboratory before using the equipment.

3 Principle

A well homogenised sample is dissolved in water and analysed with HPAEC-PAD using a anion exchange column (CarboPac PA100) and a pulsed electrochemical detector (PED).

4 Reagents

1. 2 FL (2'-fucosyllactose; trimer)
2. 3 FL (3'- fucosyllactose; impurity trimer)
3. LDFT (Lacto-di-fucotetraose or difucosyllactose; tetramer impurity)
4. Fucose, Sigma F-2252 (or equal)
5. D(+)-Galactose, Merck 1.04058 (or equal)
6. D(+)-Glucose, Merk 8227 (or equal)
7. D(+)-Lactose monohydrate, Fluka 61340 (or equal)
8. D(-)-Sorbitol, Fluka 85529 (or equal)
9. Galactitol (Dulcitol), Fluka 44590 (or equal)
10. D(-)-Mannitol, Merck 5987 (or equal)
11. Sodium Hydroxide (NaOH) 50 % solution, e.g. J. T. Baker 7067 (or equal)
12. Sodium Acetate (NaOAc), Merck 1.06268.1000
13. Water, LiChrosolv®, Merck 1.15333.2500
14. Water, HPLC-quality (e.g. Milli-Q®)
15. Membrane filter, 0,2 µm, Schleicher&Schuel ME 25 membane filter (or equal)

Toimintakäsikirja

Monitoring 2-'fucosyllactose and related compounds in the fermentation or in the purification process using high performance anion exchange chromatography with electrochemical detector (HPAEC-PAD) with Dionex PA100 column

1 Applicability

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14. Water, HPLC-quality (e.g. Milli-Q®)
15. Membrane filter, 0,2 µm, Schleicher&Schuel ME 25 membane filter (or equal)

5 Equipment

Liquid chromatograph with PED detector, e.g.

- Dionex ICS 3000 chromatographic equipment including autosampler, gradient pump and electrochemical detector
- Chromeleon software

Column: CarboPac™ PA100 (250 x 4 mm) + CarboPac PA100 Guard Column (4 x 50 mm))
Column oven temperature: 35 °C
Injection volume: 10 µl
Eluent:
A: 0.1 M NaOH solution
B: 0,1 M NaOH / 0,3 M NaOAc
C: 0.1 M NaOH solution
D: 0,1 M NaOH / 0,3 M NaOAc

Preparation of 0,1M NaOH:
Degas the water by sonication coupled with a vacuum aspirator for 5 min. Fill a 2 l volumetric flask with water almost full (appr. 1950 ml). Pipet accurately 10.4 ml of 50 % NaOH solution (from the center of the solution) to the flask and fill the flask to the mark with water. Mix the eluent with magnetic stirrer before moving it to the eluent container.

Preparation of 2,4M NaOAc:
Weigh 393,72 g NaOAc (Merck 1.06268.1000) into 2 l volumetric flask, filld to mark with H2O (Merck Wasser 1.15333.2500) and filtered with 0,2 µm filter (Schleicher&Schuel ME 25 membrane filter)

Preparation of 0,1M NaOH / 0,3M NaOAc:
10,4 ml 50% NaOH + 250 ml 2,4M NaOAc measure into a 2 l volumetric flask, fill to the mark with Milli-Q water (water kept in ultrasonic bath with suction for 10 minutes before using)

Used gradient:

Time (min)	Flow (ml/min)	% A	% B	% C	% D
0.00	1.0	49,9	0,2	49,9	0,2
20.00	1.0	49,9	0,2	49,9	0,2
20.10	1.0	37,5	12,5	37,5	12,5
30.00	1.0	37,5	12,5	37,5	12,5
30.10	1.0	49,9	0,2	49,9	0,2
45.00	1.0	49,9	0,2	49,9	0,2

Data collection rate: 1.0 Hz

WaveformName: "carbohydrate (triple potential)"
The form of PED voltage impulse

Time (s)	Voltage (V)	GainRegion
0.00	0.05	
0.20	0.05	Begin
0.40	0.05	End
0.41	0.75	
0.60	0.75	
0.61	-0.15	
1.00	-0.15	

6 Sampling

The sample sent to the laboratory should be representative.

7 Performance

7.1 Preparation of the standard solutions

Prepare separately for each standard compound different stock solutions. Weigh accurately the component into a 50 ml flask. Dissolve the component carefully in water.
Fill the flask to the mark, and mix well. Dilute stock solutions to working range (Table 1)
Use standards (Std A,C,D,E,F,G) for one-point calibration and standard B (Std B) as reference standard.

Table 1. Suggestion for preparation of the standard solutions (Std). Purity calculations are noticed in calculations.

	Component	Stock (mg/50 ml)	Dilute Stock	Concentr. (mg/1000ml)
Std A	2-`Fucosyl lactose (2`-FL)	10.0	1.0ml/10ml	20.0
Std B	2-`Fucosyl lactose (2`-FL)	10.0	1.0ml/10ml	20.0
Std C	3-Fucosyl lactose (3-FL)	10.0	0.5ml/20ml	5.0
Std D	Lactodifucotetraose (LDFT)	10.0	0.5ml/20ml	5.0
Std E	Lactose	10.0	0.5ml/50ml	2.0
Std F	Glucose	10.0	0.5ml/100ml	1.0
	Fucose	10.0	0.5ml/100ml	1.0
	Sorbitol	10.0	0.5ml/100ml	1.0
	Mannitol	10.0	0.5ml/100ml	1.0
Std G	Galactitol	10.0	0.5ml/100ml	1.0

Standard stock solution can be stored in a freezer (-18 °C) for one year.

7.2 Preparation of the sample solution

Weigh the sample accurately into the flask and fill the flask to the mark. Dilute the sample solution with water so that the 2FL content of the prepared solution is in range 1-200 mg/l.

7.3 Procedure

Perform a system suitability test when the HPLC system is used for the first time or some condition in the system is changed e.g. after the working electrode has changed:

1. Let the HPLC system stabilize for about half an hour.
2. Inject one of the standard solutions (e.g. standard solution A) 6 times. The relative standard deviation of the peak response (area) must be ≤ 3 %
3. Inject a prepared control standard. Pay attention to the form of peaks, retention time, integration.

Everyday runs:

1. Inject water. The chromatogram should be a straight line.
2. For one-point calibration inject the standard solution (Std A) 3 times.
3. Inject the standard solutions (Std C,D,E, F)
4. Inject the control standard (Std B)
5. Inject 8 samples
6. Inject the standard solutions (Std A,C,D,E, F)
7. The last injection is the control standard (Std B)

1. 8 Results

8.1 Calculations

The results are calculated with an external standard method using peak area.
An example of calculations is presented below.

$$(\%\, LW) = \frac{R_{sample} \cdot W_{std}(mg) \cdot Purity_{std} \cdot V_{sample}(ml)}{R_{std} \cdot V_{std}(ml) \cdot W_{sample}(mg)} \cdot 100\%$$

LW = Liquid weight or natural weight
R_{sample} = response of the analyte in sample solution (area)
R_{std} = response of the analyte in standard solution (area)

W_{sample} = amount of the sample weighed (mg)
W_{std} = amount of the analyte weighed in standard solution (mg)
V_{sample} = volume of the volumetric flask sample was weighed in (ml)
V_{std} = volume of the volumetric flask standard was weighed in (ml)
$Purity_{std}$ = purity of the standard compound content calculated from weighed reference material taking into account the moisture and purity of the reagent.

$$Analyte\,(\%\,DS) = Analyte\,(\%\,LW) \cdot \frac{100}{DS_{sample}(\%)}$$

DS_{sample} = dry substance of the sample

8.2 Expression of the result

The results are given with one decimal as percent of dry substance (% d.s.) if it is known or percent of liquid weight (% liquid weight). For process follow-up, it is also possible to report the results as area-%.

9 Validation of the method

The linearity of the method has been verified in the range 1-200mg/l. The accuracy and selectivity of the method has not been verified.

10 References

Safety Evaluation of 2'-Fucosyllactose for Use in Term Infant Formulas and Toddler Formulas, Appendix F4: Identification and Confirmation of Jennewein 2'-FL by HPAEC-PAD. GRAS Notice (GRN) No. 571

http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm

11 Source of error

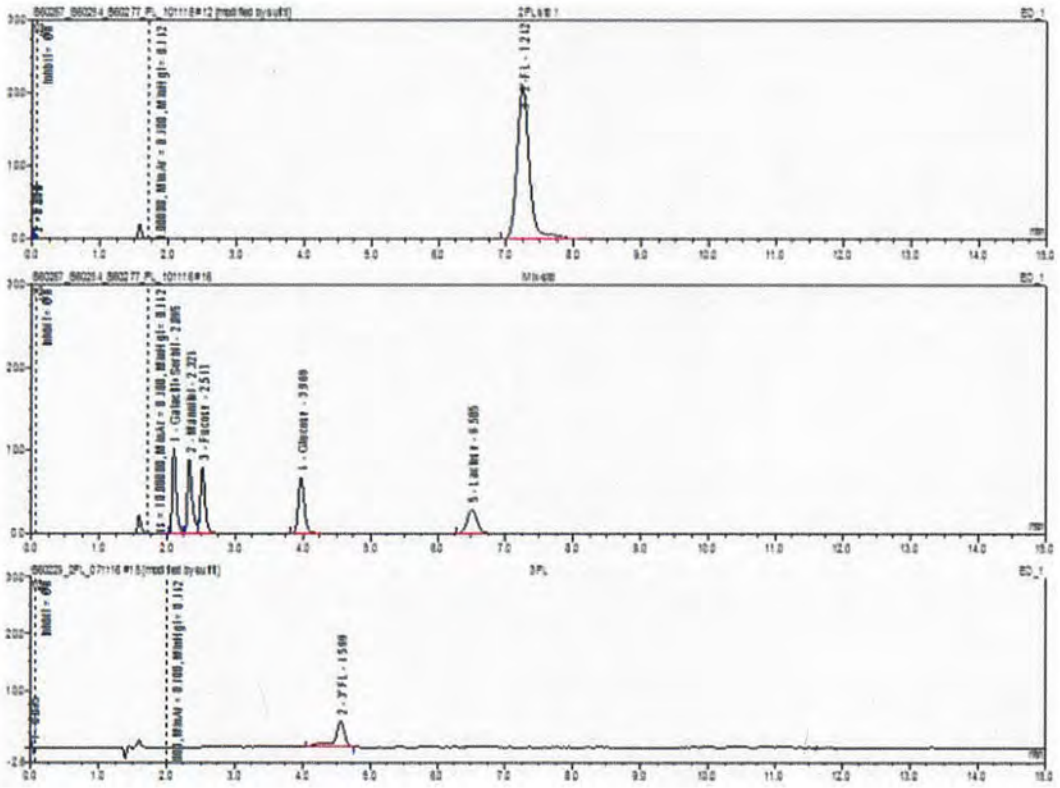
- Error in injection -> Contact the maintenance service of your equipment
- Dirty electrode causes decreasing response, poor repeatability, increased baseline noise. The old reference electrode causes ghoast peaks in the chromatogram -> Clean or change the electrode and/or according to the manufacturer's instructions
- Air bubbles in the system -> Clean the sinters in eluent container, remove the air from the eluent etc.
- Impure standards, wrong dry substance and wrong integration parameters may also result in errors.
- Galactose and glucose are coeluting
- Galactitol and Sorbitol are coeluting

- Lactose and Lactulose are coeluting

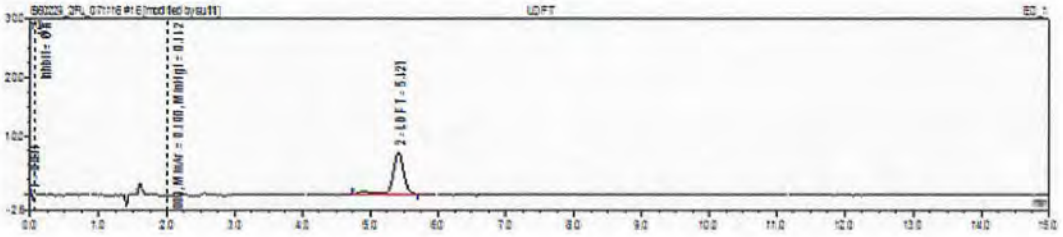
12 Chromatograms

Chromatograms of standards:

2-FL	rt 8.9 min
Galactitol+Sorbitol	rt 2.8 min
Mannitol	rt 3.0 min
Fucose	rt 3.2 min
Glucose	rt 4.9 min
Lactose	rt 8.0 min
3-FL	rt 4.6 min
Difucosyllactose (LDFT)	rt 5.4 min



0524 Monitoring 2-'fucosyllactose and related compounds in the fermentation or in the purification process using high



SISÄLLYSLUETTELO	02. TYÖOHJEET\02:02 ANALYYSIOHJEET\02.02.03 LC\02.02.03.03 PED
VIITESTANDARDI	SFS-EN ISO/IEC 17025
VERSIO/KOODI	1 / 0524
VOIMASSA	27.01.2017
LAATIJA	SIIRI VIIKARI
TARKASTETTU	26.01.2017 13:01 JOHANNA AVELLAN
HYVÄKSYTTY	26.01.2017 15:00 SIIRI VIIKARI

Toimintakäsikirja

Otsikko	**Determination of moisture of dry powder sample by Coulometric Karl-Fisher titration**		
Sisällysluettelo	**02. TYÖOHJEET\02.02 Analyysiohjeet\02.02.01 EO**		
		Asiakirjatyyppi	Analyysiohje\EO
Viitestandardi	SFS-EN ISO/IEC 17025	Versio / Koodi	1 / 0525
Organisaatio	DuPont Nutrition and Health Manufacturing Technology Kantvik	Voimassa	19.11.2016
Laatija	Siiri Viikari		
Tarkastettu	18.11.2016 Pertti Sarela		
Hyväksytty	18.11.2016 Siiri Viikari	Edellinen versio	/

Determination of moisture of dry powder sample by Coulometric Karl-Fisher titration

1 Application

The method is applied to the determination of moisture from dry powder samples.

2 Safety

Reagents used for this analysis are harmfull. Read MSDS before the work.
The methanol and Karl-Fischer reagent are irritating, avoid skin contact. Methanol is poisonous.
Used chemicals have to be disposed according to local instructions.
Use fume hood while working with this method. Allways use appropriate gloves (e.g TouchNTuff) while changing the chemicals.
Be carefull while working with the glassware. Check the glassware before using it.

3 Principle

The moisture of the sample is extracted to dry methanol and iodine is produced electrochemically in the titration vessel. The voltametric method is used for detection of the end point: an alternating current to a pair of indicator electrodes. The quantitative relationship existing between the electric charge passed and the amount of iodine produced is used for the precise dosing of iodine.

4 Reagents

1. Karl Fischer Reagent, Merck 9255.0500 or equivalent

2. Water Standard solid 5%, Merck 1.129394.
3. Water Standard Solution 5000 ppm, Merck 2959

5 Equipment

1. Coulometric KF titrator Metrohm 684 equipped with titration unit and a mixer.

2. Analytical balance

6 Sampling

The samples should be stored well closed.

7 Performance

The titration vessel and the electrodes are washed with water and rinsed with methanol or acetone. Detergents can be used to make the cleaning effective. The platinum mesh of the generator electrode must be handled carefully, it is easily broken. The vessel and electrodes are dried in a desiccator or in warm air (max. 70°C). Higher temperatures must be avoided because they can be damage the plastic cables. Cleaning is carried out whenever the drift of the instrument keeps being higher than indicated in the manual. After cleaning the vessel is filled with 80-90 ml of reagent.

7.1 Standardisation of the equipment

The performance of the instrument is checked before every use though once a day is adequate with a water standard using 2 min extraction time and 10 s delay.

Following standards are in use:

1. Solid 5 % water standard, accurate water content is marked on the label.

2. Liquid 5000 ppm standard (5 mg H_2O / ml, 0.6173 w-%)

The result of the standard may differ 4 % of the given value (5.201 ± 0.208 %, 0.6173 ± 0.025 %). If the result differs more then the

moisture content of the standard, it should be redetermined. If result is still not within the limits, the vessel and the electrodes must be cleaned as described in section 5.1

7.2 Procedure

Weigh accurately 0.04-0.1 g of the dry powder sample into the vessel. Extract for 2 minutes with 10 s delay. Read the result and calculate the water content with two decimals.

8 Results

The final result of sample is calculated as a mean of three parallel measurements. The result is given as % with two decimals.

9 Validation

The Method is not validated.

SISÄLLYSLUETTELO	02. TYÖOHJEET\02.02 ANALYYSIOHJEET\02.02.01 EO
VIITESTANDARDI	SFS-EN ISO/IEC 17025
VERSIO/KOODI	1 / 0525
VOIMASSA	19.11.2016
LAATIJA	SIIRI VIIKARI
TARKASTETTU	18.11.2016 09:30 PERTTI SARELA
HYVÄKSYTTY	18.11.2016 09:49 SIIRI VIIKARI

analytical method

Moisture determination with volumetric Karl Fischer titration

Version: 01; Issue date: 05/2016

The determination of moisture in the sample is based upon the quantitative reaction of water with sulfur dioxide and iodine in a suitable anhydrous medium in the presence of a base with sufficient buffering capacity. The water in the sample is extracted to anhydrous methanol in a titration vessel. The extracted water in the solvent reacts stoichiometrically with the iodine in the titrant. The titration end point is determined by electrometric indication method. The volume of the used Karl Fischer titrant and the known sample weight in the titration vessel are used to calculate the moisture content of the sample. The analysis is performed with automated titration instrument.

Finnfeeds Finland Oy, a subsidiary of E.I. du Pont de Nemours and Company

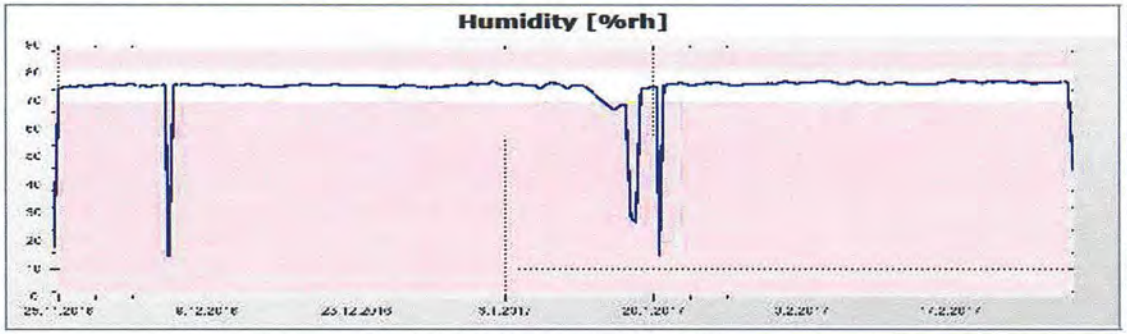
Satamatie 2,FIN-21100 Naantali, Finland
Tel +358 10 431 020, Fax +358 10 4318 333

Domicile Naantali, Business ID 1508199-2, Vat No. FI15081982

ANNEX 2:

Datalogger reports of temperature and humidity controlled at 12-h intervals.
Climate cabinet at 40 °C / 75 % RH

Report
Filename: D407_2 2802.LOG / Device: D.407-2 / Serial: 815050425



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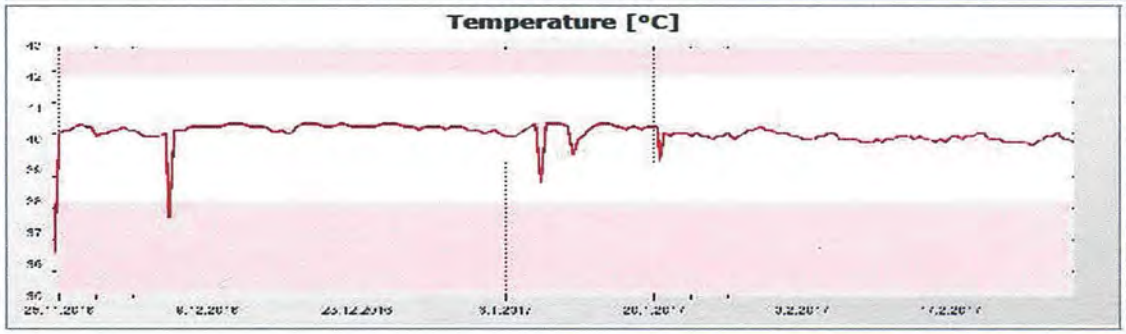
Begin	24.11.2016 13:46:05
End	28.2.2017 13:31:15
Min	14,36 5.12.2016 9:39:45
Max	77,40 17.2.2017 1:31:15
Average	74,117
STDEV	9,193



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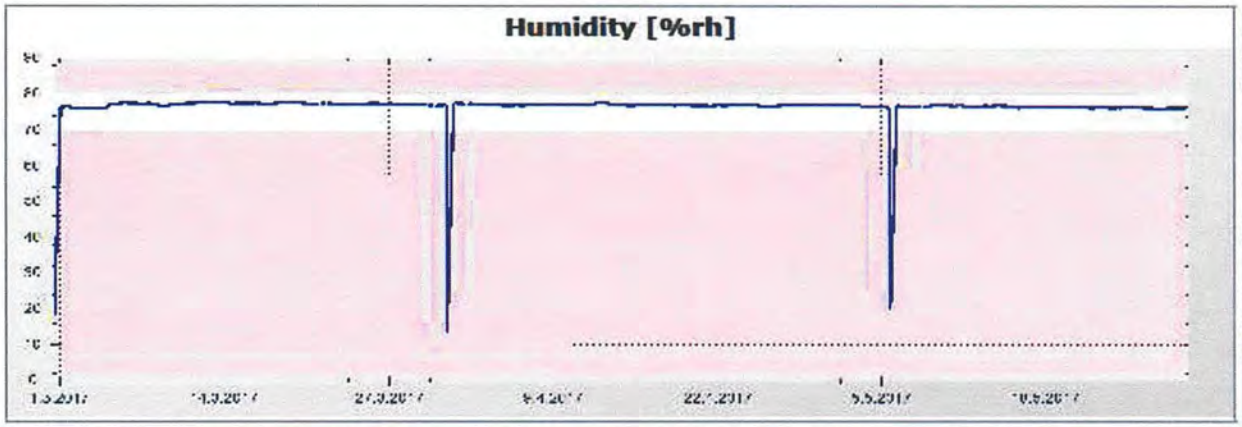
End	28.2.2017 13:31:15
Min	36,40 24.11.2016 13:46:05
Max	40,50 27.11.2016 1:46:05
Average	40,184
STDEV	0,409
MKT	40,191

HW4, Version V3.7.0.15327

Report
Filename: D407-2 29052017.LOG / Device: D.407-2 / Serial: 815050425



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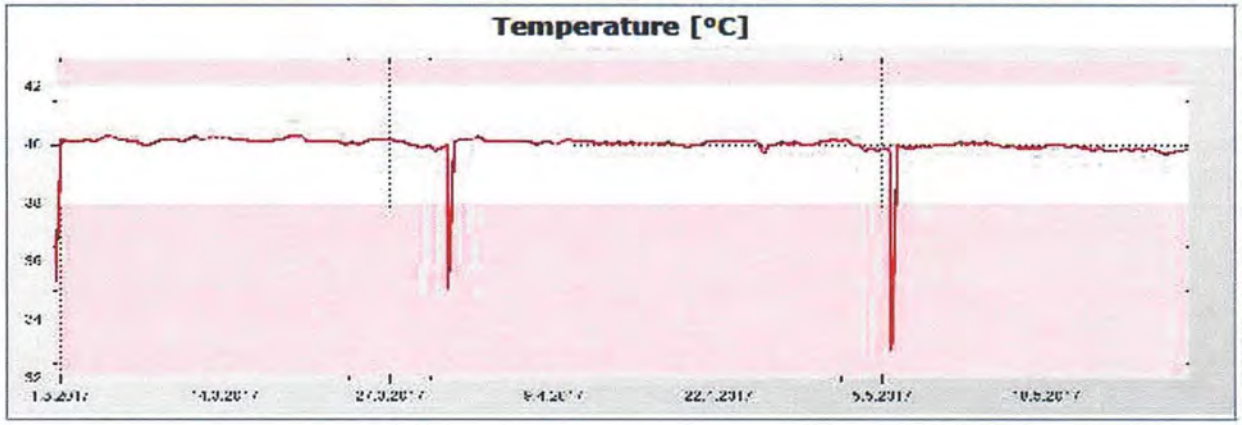
Average	76,017
STDEV	7,649



Statistics	
Count	182
Interval	12:00:00
Begin	28.2.2017 13:51:00
End	29.5.2017 5:40:10
Min	32,90 5.5.2017 17:40:10
Max	40,30 4.3.2017 13:51:00
Average	39,954
STDEV	0,746
MKT	39,976

HW4, Version V3.7.0.15327

ANNEX 3

Detailed measurement results

T=0 WEEKS

no	sample	Moisture %	Galactitol+ Sorbitol area-%	Mannitol area-%	Fucose area-%	Glucose + Galactose area-%	3' FL area-%	LDFT area-%	Lactose area-%	2'-FL area-%	rt 8.0- area-%	Sum area-%
1A	F13/1	2,2	1,0	< 0,2	0,2	< 0,2	< 0,2	7,9	6,6	82,9	1,2	100
1B	t=0	(Outsourced)	1,0	< 0,2	0,2	< 0,2	< 0,2	7,7	6,5	83,1	1,3	100
1C			1,0	< 0,2	0,2	< 0,2	< 0,2	7,7	6,5	83,1	1,3	100
	Avg		1,0		0,2			**7,8**	**6,5**	**83,0**	1,3	100
	SD		0,0		0,0			0,1	0,1	0,1	0,0	
	RSD-%		0,6		3,5			1,1	0,8	0,2	1,4	
2A	F13/2a	2,8	1,1					7,4	6,9	84,6		100
2B	t=0	(Outsourced)	1,0					7,6	6,5	84,9		100
2C			0,9					8,0	6,6	84,6		100
	Avg		1,0					**7,7**	**6,7**	**84,7**		100
	SD		0,1					0,3	0,2	0,2		
	RSD-%		11,2					3,7	3,2	0,2		
3A	F13/3	5,0	1,0	< 0,2	< 0,2	< 0,2	< 0,2	7,6	6,5	83,3	1,2	100
3B	t=0	(Outsourced)	1,0	< 0,2	< 0,2	< 0,2	< 0,2	7,7	6,5	83,4	1,2	100
3C			1,0	< 0,2	< 0,2	< 0,2	< 0,2	7,6	6,6	83,2	1,3	100
	Avg		1,0					**7,6**	**6,5**	**83,3**	1,2	100
	SD		0,0					0,1	0,0	0,1	0,1	
	RSD-%		1,0					1,0	0,7	0,1	4,7	

T= 1 WEEK

no	sample	Moisture %	Galactitol+ Sorbitol area-%	Mannitol area-%	Fucose area-%	Glucose + Galactose area-%	3' FL area-%	LDFT area-%	Lactose area-%	2'-FL area-%	rt 8.0- area-%	Sum area-%
4A	F13/1, 40C / 75%RH	6,6	1,1		0,2			7,4	6,8	82,6	2,0	100
4B	t=1	6,7	1,0		0,6			7,9	6,6	83,0	0,9	100
4C		6,8	0,9		0,1			7,2	6,3	84,5	0,9	100
	Avg	**6,7**	1,0		0,3			**7,5**	**6,6**	**83,4**	1,2	100
	SD	0,1	0,1		0,3			0,3	0,2	1,0	0,6	
	RSD-%	1,1	7,0		95,2			4,6	3,3	1,2	50,3	
5A	F13/2a, 40C / 75%RH	5,8	1,0					8,1	7,0	82,7	1,3	100
5B	t=1	5,8	0,8					7,6	6,5	84,0	1,1	100
5C		5,8	0,8					7,6	6,7	83,7	1,2	100
	Avg	**5,8**	0,9					**7,8**	**6,7**	**83,5**	1,2	100
	SD	0,0	0,1					0,3	0,3	0,7	0,1	
	RSD-%	0,6	11,1					3,4	4,0	0,8	5,9	
6A	F13/3, 40C / 75%RH	5,6	1,2					7,1	6,4	84,2	1,1	100
6B	t=1	5,5	1,0					7,4	6,8	83,3	1,5	100
6C		5,6	0,9					8,0	7,0	83,3	0,8	100
	Avg	**5,6**	1,0					**7,5**	**6,7**	**83,6**	1,1	100
	SD	0,0	0,2					0,4	0,3	0,5	0,3	
	RSD-%	0,4	17,4					5,9	4,1	0,6	30,8	

T= 4 WEEKS

no	sample	Moisture %	Galactitol+ Sorbitol area-%	Mannitol area-%	Fucose area-%	Glucose + Galactose area-%	3' FL area-%	LDFT area-%	Lactose area-%	2'-FL area-%	rt 8.0- area-%	Sum area-%
4A	F13/1, 40C / 75%RH	7,8	0,9		< 0,2			7,0	6,3	84,5	1,2	100
4B	t=4	7,4	0,9		< 0,2			6,8	6,2	85,1	1,0	100
4C		7,5	0,8		< 0,2			7,0	6,0	85,0	1,1	100
	Avg	**7,6**	0,8					**7,0**	**6,2**	**84,9**	1,1	100
	SD	0,2	0,0					0,1	0,1	0,3	0,1	
	RSD-%	2,6	1,8					1,8	2,3	0,3	9,7	
5A	F13/2a, 40C / 75%RH	8,7	0,9		< 0,2			7,3	6,4	84,2	1,1	100
5B	t=4	8,7	0,9		< 0,2			7,1	6,4	84,4	1,1	100
5C		8,3	0,8		< 0,2			7,4	6,3	84,3	1,2	100
	Avg	**8,6**	0,8					**7,3**	**6,4**	**84,3**	1,1	100
	SD	0,2	0,0					0,1	0,1	0,1	0,1	
	RSD-%	2,8	5,6					1,7	0,8	0,1	4,5	
6A	F13/3, 40C / 75%RH	9,5	0,9					7,3	6,5	84,1	1,2	100
6B	t=4	9,2	0,9					7,1	6,5	84,1	1,4	100
6C		9,1	0,9					7,4	6,6	83,9	1,3	100
	Avg	**9,3**	0,9					**7,3**	**6,5**	**84,0**	1,3	100
	SD	0,2	0,0					0,2	0,0	0,1	0,1	
	RSD-%	2,4	1,7					2,1	0,4	0,1	5,7	

T= 8 WEEKS

no	sample	Moisture %	Galactitol+ Sorbitol area-%	Mannitol area-%	Fucose area-%	Glucose + Galactose area-%	3' FL area-%	LDFT area-%	Lactose area-%	2'-FL area-%	rt 8.0- area-%	Sum area-%
		volumetric KF										
4A	F13/1, 40C / 75%RH	6,1	0,9					7,9	6,5	84,7	< 0,2	100
4B	t=8	5,6	1,1					8,3	7,0	83,6	< 0,2	100
4C		4,9	1,0					7,3	7,1	84,5	< 0,2	100
		5,2										
	Avg	**5,5**	1,0					**7,9**	**6,9**	**84,3**		100
	SD	0,5	0,1					0,5	0,4	0,6		
	RSD-%	9,4	7,6					6,5	5,2	0,7		
		volumetric KF										
5A	F13/2a, 40C / 75%RH	7,9	1,1		< 0,2			7,6	7,6	83,8	< 0,2	100
5B	t=8	8,5	1,0		< 0,2			7,4	7,1	84,3	< 0,2	100
5C		8,6	0,9		< 0,2			8,7	7,2	82,8	< 0,2	100
		7,8										
	Avg	**8,2**	1,0					**7,9**	**7,3**	**83,6**		100
	SD	0,4	0,1					0,7	0,3	0,8		
	RSD-%	5,0	10,6					9,2	3,8	0,9		
		volumetric KF										
6A	F13/3, 40C / 75%RH	7,8	7,8	7,8				6,9	6,4	85,6	< 0,2	114
6B	t=8	7,6	7,6	7,6				7,1	7,5	84,4	< 0,2	114
6C		7,9	7,9	7,9				7,3	6,9	84,9	< 0,2	115
	Avg	**7,7**	7,7					**7,1**	**6,9**	**85,0**		107
	SD	0,2	0,2					0,2	0,6	0,6		
	RSD-%	2,0	2,0					2,3	8,1	0,7		

T= 13 WEEKS

no	sample	Moisture %	Galactitol+ Sorbitol area-%	Mannitol area-%	Fucose area-%	Glucose + Galactose area-%	3' FL area-%	LDFT area-%	Lactose area-%	2'-FL area-%	rt 8.0- area-%	Sum area-%
		volumetric KF										
4A	F13/1, 40C / 75%RH	4,8	1,1		< 0,2			7,3	7,0	83,8	0,7	100
4B	t=13	4,5	1,0		< 0,2			7,5	6,8	83,5	1,1	100
4C		4,6	1,2		< 0,2			7,6	6,8	83,3	1,1	100
		4,4										
		4,3										
	Avg	**4,5**	1,1					**7,4**	**6,9**	**83,5**	1,0	100
	SD	0,2	0,1					0,1	0,1	0,3	0,2	
	RSD-%	4,0	9,9					1,8	2,1	0,3	22,4	
		volumetric KF										
5A	F13/2a, 40C / 75%RH	6,5	1,0		< 0,2			7,5	7,0	83,3	1,1	100
5B	t=13	6,4	1,0		< 0,2			7,5	7,1	82,8	1,4	100
5C		6,1	1,1		< 0,2			7,0	7,3	83,2	1,2	100
	Avg	**6,3**	1,1					**7,3**	**7,1**	**83,1**	1,2	100
	SD	0,2	0,1					0,3	0,1	0,3	0,2	
	RSD-%	3,4	5,5					3,8	1,6	0,3	12,9	
		volumetric KF										
6A	F13/3, 40C / 75%RH	7,2	1,1					7,4	8,2	82,4	1,0	100
6B	t=13	7,6	1,1					7,2	8,3	82,4	1,1	100
6C		7,0	1,0					7,6	8,2	82,2	1,1	100
		6,9										
		6,8										
	Avg	**7,1**	1,0					**7,4**	**8,2**	**82,3**	1,1	100
	SD	0,3	0,0					0,2	0,1	0,1	0,1	
	RSD-%	4,3	4,6					2,7	0,8	0,1	6,7	

T= 26 WEEKS

no	sample	Moisture %	Galactitol+ Sorbitol area-%	Mannitol area-%	Fucose area-%	Glucose + Galactose area-%	3' FL area-%	LDFT area-%	Lactose area-%	2'-FL area-%	rt 8.0- area-%	Sum area-%
		volumetric KF										
4A	F13/1, 40C / 75%RH	3,5	0,9		< 0,2			7,61	6,93	83,57	0,80	100
4B	t=26	3,1	1,0		< 0,2			7,42	6,75	83,55	1,06	100
4C		3,4	0,9		< 0,2			7,49	6,70	83,71	1,05	100
	Avg	**3,4**	1,0					**7,5**	**6,8**	**83,6**	1,0	100
	SD	0,2	0,1					0,1	0,1	0,1	0,1	
	RSD-%	7,0	5,8					1,3	1,8	0,1	15,2	
		volumetric KF										
5A	F13/2a, 40C / 75%RH	4,1	0,9		< 0,2			7,3	7,2	83,4	1,0	100
5B	t=26	3,3	0,8		< 0,2			7,8	6,9	83,1	1,3	100
5C		4,6	0,9		< 0,2			7,6	6,9	83,2	1,3	100
		3,3										
		3,9										
	Avg	**3,8**	0,9					**7,6**	**7,0**	**83,2**	1,2	100
	SD	0,6	0,0					0,2	0,2	0,2	0,2	
	RSD-%	14,4	5,6					3,3	2,6	0,2	13,0	
		volumetric KF										
6A	F13/3, 40C / 75%RH	5,6	0,8					7,65	7,06	83,44	1,03	100
6B	t=26	5,6	0,8					7,38	6,9	83,89	1,06	100
6C		5,3	0,9					7,28	7,31	83,44	1,12	100
	Avg	**5,5**	0,8					**7,4**	**7,1**	**83,6**	1,1	100
	SD	0,2	0,0					0,2	0,2	0,3	0,0	
	RSD-%	3,1	5,5					2,6	2,9	0,3	4,3	