# JHeimbach LLC



April 30, 2020

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

Dear Dr. Carlson:

Pursuant to 21 CFR Part 170, Subpart E, DuPont Nutrition and Biosciences, through me as its agent, hereby provide notice of a claim that the addition of 3-fucosyllactose produced by genetically engineered *Escherichia coli* K12 MG1655 to nonexempt term infant formula, formula intended for young children 12 months of age and older, and other foods and beverages that are consumed by toddlers under 3 years of age and by the general US population aged 3 years and above is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because DuPont Nutrition and Biosciences has determined that the intended use is generally recognized as safe (GRAS) based on scientific procedures.

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

If you have any questions regarding this notification, please feel free to contact me at 804-742-5543 or jh@jheimbach.com.

Sincerely,

[redacted]

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

Encl.

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

# COMPREHENSIVE GRAS ASSESSMENT

**for the proposed use of**

## 3-FUCOSYLLACTOSE

**in**

**Nonexempt term infant formula, formula intended for young children 12 months of age and older, and other foods and beverages consumed by toddlers under 3 years of age and by the general US population aged 3 years and above**

**April 2020**

**DuPont Nutrition & Biosciences**

**Edited by**
**JHeimbach LLC**

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# PART 1. SIGNED STATEMENTS AND CERTIFICATIONS

## 1.1. GRAS Notice Submission

In accordance with 21 CFR 170.255, Danisco USA Inc. (dba DuPont Nutrition & Biosciences [DuPont]) submits this GRAS notice through its agent James T. Heimbach, president of JHeimbach LLC, for 3-fucosyllactose (3FL) produced by a genetically engineered *Escherichia coli* K12 MG1655 production strain.

## 1.2. Name and Address of Notifier

DuPont Nutrition & Biosciences
DuPont Experimental Station - E320
200 Powder Mill Road
Wilmington, DE 19803

Notifier Contact
Angela Lim
Global Regulatory Strategy Lead (HMOs & Food Protection)
Tel: 302-695-6786
Angela.Lim@DuPont.com

Agent Contact
James T. Heimbach, Ph.D., F.A.C.N.
President
JHeimbach LLC
923 Water Street #66
Port Royal VA 22535
Tel: 804-742-5543
jh@jheimbach.com

## 1.3. Name of the Notified Substance

3-fucosyllactose (3FL)

## 1.4. Intended Conditions of Use of the Notified Substance

3FL is intended be used as a food ingredient in nonexempt term infant formulas, formula intended for young children 12 months of age and older, and other foods and beverages that are consumed by toddlers under 3 years of age and by the general US population aged 3 years and above. Proposed use levels range from 0.2 g/serving (2.0 g/L) in infant formula and formula intended for young children 12 months of age and older to levels ranging from 0.14 to 4.0 g/serving (1.2 to 40 g/kg) in other foods and beverages.

## 1.5. Statutory Basis for Conclusion of GRAS status

DuPont has concluded that the notified substance, 3-fucosyllactose (3FL), as described herein is generally recognized as safe (GRAS) under the conditions of its intended use. This GRAS conclusion was reached through scientific procedures and in concert with the views of a panel of experts who

are qualified by scientific training and experience to evaluate the safety of substances added to foods, in accordance with 21 CFR 170.30(a) and (b).

## 1.6. Claim of Exclusion from the Requirement for Premarket Approval

Based upon DuPont's GRAS conclusion as stated in Part 1, Section 1.5 above, it is DuPont's view that 3FL is not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act.

## 1.7. Availability of Data and Information

DuPont agrees to make available the data and information that are the basis for DuPont's 3FL GRAS conclusion available for review and copying at FDA's request during customary business hours at the office listed in Part 1, Section 1.2 above. A complete copy of the data and information will be provided to FDA upon request.

## 1.8. Disclosure under the Freedom of Information Act

This GRAS notice does not contain data and information that are exempt from disclosure under the Freedom of lnformation Act (FOIA), USC 552.

## 1.9. Certification Statement

To the best of our knowledge, this dossier/notice presents 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 intended use of 3FL.

## 1.10. FSIS Statement

Not applicable.

## 1.11. Name, Position, and Signature of Notifier

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

# PART 2. IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS AND EFFECT

## 2.1. Substance Identity

**Common name:** 3-Fucosyllactose

**Abbreviated names:** 3FL, 3-FL

**Alternative names:** 3-O-Fucosyllactose

**IUPAC name[1]:** 6-Deoxy-α-L-galactopyranosyl-(1->3)-β-D-galactopyranosyl-(1->4)-D-glucopyranose

**CAS number:** 41312-47-4

**Chemical formula:** $C_{18}H_{32}O_{15}$

**Molecular weight:** 488.4 g/mol

**Structural formula:**

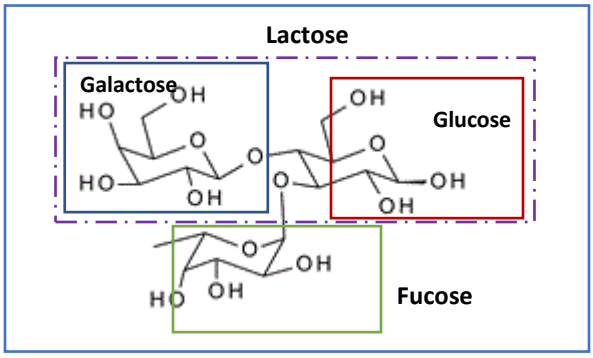



**Figure 1: Structural Formula of 3FL**

## 2.2. Description

3FL is a milk oligosaccharide composed of a lactose core of D-glucose and D-galactose units linked via an α(1-3) bond to L-fucose. It is a white to ivory-colored powder that is soluble in water.

Originally identified and isolated from human breast milk in the mid-1950s (Kunz, 2012), 3FL belongs to a group of complex carbohydrates described as human milk oligosaccharides (HMOs). HMOs are the third largest component in breast milk, totaling on average 12.9 g/L in mature milk and 20.9 g/L at 4 days post-partum (Andreas et al., 2015).

The 3FL under discussion in this monograph is produced by fermentation using a modified *E. coli* K12 host production strain. Nuclear magnetic resonance spectroscopy ($^1$H NMR) was used to compare three batches of *E. coli*-derived 3FL to 3FL isolated from human milk[2]. The $^1$H NMR spectra (Figure 2) show that all major well-resolved signals in the spectra of the 3FL samples are identical,

[1] CSID:141824, http://www.chemspider.com/Chemical-Structure.141824.html (accessed 21:55, Sep 18, 2019)

[2] Sourced from IsoSep AB, Lot No.:1042-092, purity >95%

indicating that there is no significant difference between 3FL derived from *E. coli* K12 MG1655 sINB008971 and 3FL isolated from human milk. Additionally, the recorded $^{1}$H NMR spectra of the samples are identical to spectral data reported in the published literature (van Leeuwen et al., 2014).

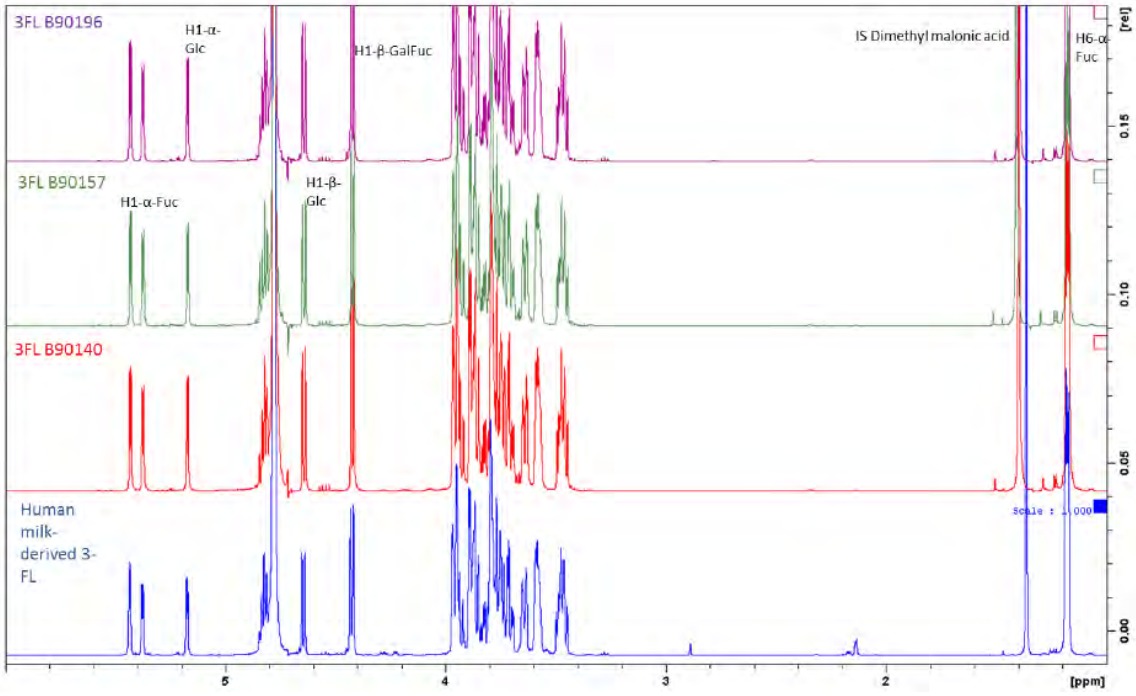



**Figure 2: Comparison of 1H NMR Spectra of 3FL Batches to Human Milk-Derived 3FL**

The 3FL was further characterized using hydrophilic interaction liquid chromatography (HILIC) with refractive index detection (HILIC-RID) and with mass spectrometric detection (HILIC-MS) along with the relevant reference standards. The data showed that, in addition to the 3FL molecule, lactose, galactose/glucose and other carbohydrates are present as minor components and/or as residuals from the downstream processing step. The residual components were identified as mono-, di- and trimeric carbohydrates with a polyol nature.

The engineered *E. coli* K12 MG1655 production strain is removed during the downstream processing step and is not present in the 3FL product. The absence of the production host and any residual rDNA is confirmed by test as shown below.

## 2.3. Method of Manufacture

### 2.3.1. Process Description

DuPont's 3FL production process can be divided 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 genetically engineered *E. coli* K12 MG1655 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 medium is supplemented with other nutrients such as trace minerals, vitamins, and amino acids. These include food-grade ammonium chloride, ammonium sulfate, potassium phosphate, 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 betaine. Food-grade processing aids such as antifoam and pH control agents may also be used in the process. All ingredients and processing aids are permitted for direct addition to foods as GRAS ingredients and/or food additives.

The fermentation stage is maintained under controlled temperature and pH conditions to optimize growth of the production strain and its expression of 3FL. 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 (outlined in Figure 3) serves to purify and selectively concentrate the 3FL 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 modified *E. coli* K12 cell biomass, endotoxins, large molecules (e.g. proteins from the fermentate), and antifoam; this is achieved using microfiltration and/or ultrafiltration. The cell-free fermentate may be treated with the lactase enzyme beta-galactosidase (EC 3.2.1.23) to hydrolyze residual lactose prior to the nanofiltration step, which removes water, inorganic salts and small carbohydrate molecules. The resulting concentrated fermentate is passed through ion exchange columns and overactive carbon where small molecules (e.g. organic acids, inorganic salts) formed/used in the fermentation stage and other compounds that may cause off-color and off-flavor are separated out. Potential microbial contaminants are then eliminated via sterile filtration and the resulting output is concentrated by evaporation/nanofiltration to further remove excess water and facilitate crystallization of the 3FL product. Depending upon customer preference, the resulting crystals may be commercialized as is or be dissolved in water and spray-dried. All processing aids used in the post fermentation processing stage, including but not limited to beta-galactosidase, ion exchange resins, filter aids, and regenerating solutions, are food-grade aids permitted for use in food processing.

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 manufacturing sites operate in accordance with current good manufacturing practice (cGMP) and/or Global Food Safety Initiative (GFSI) certification requirements and comply with the requirements of the Food Safety Modernization Act.

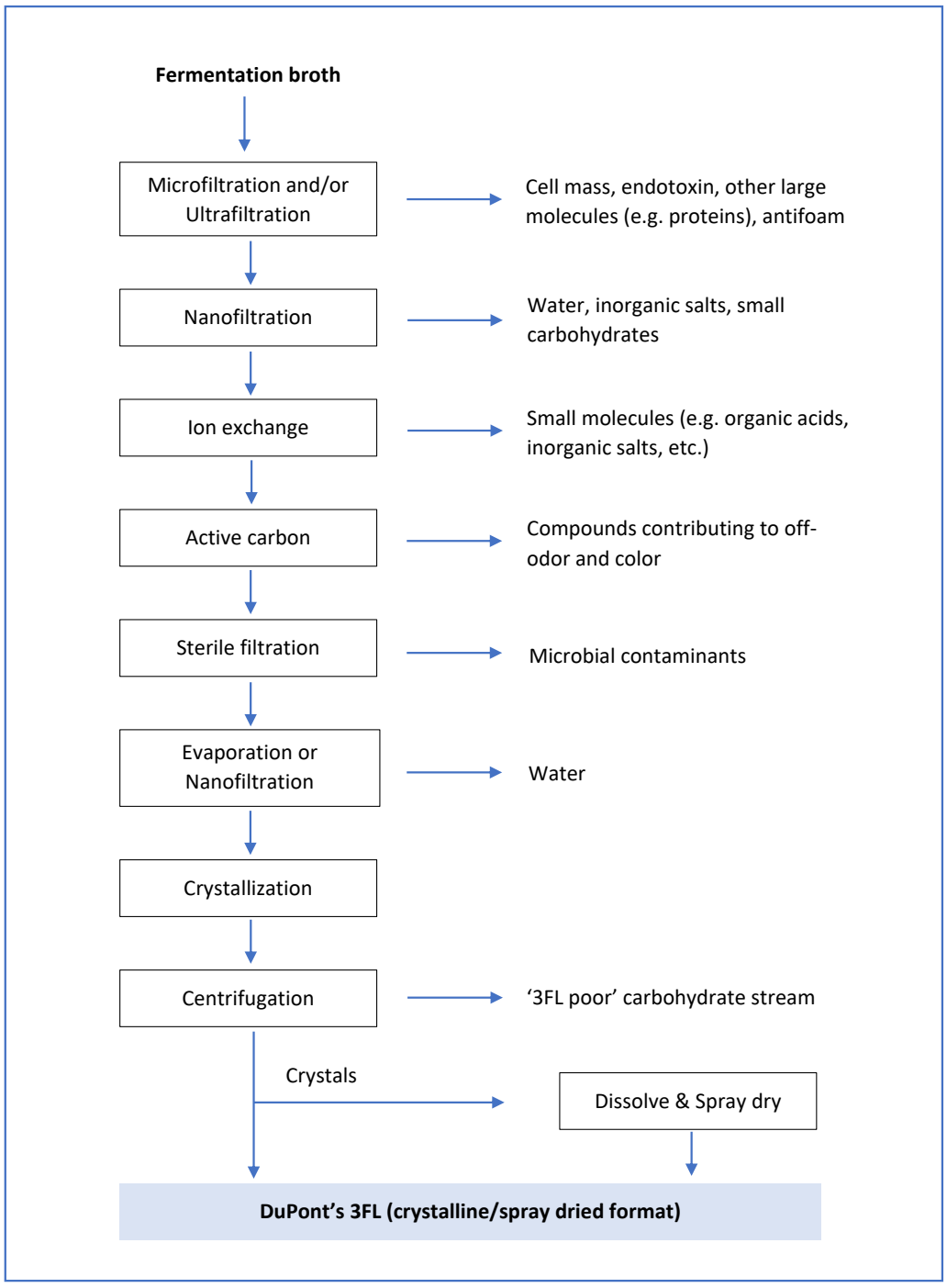



**Figure 3: 3FL Process Flow Diagram (Post Fermentation)**

## 2.3.2. Production Microorganism

**Production strain:** *E. coli* K12 MG1655 INB008971

The production strain, a genetically engineered *E. coli* K12 MG1655 strain, was constructed to produce high amounts of 3FL in large-scale industrial processes. This process makes use of endogenous production of GDP-fucose and the transfer of this nucleotide-activated sugar to the substrate lactose to result in 3FL.

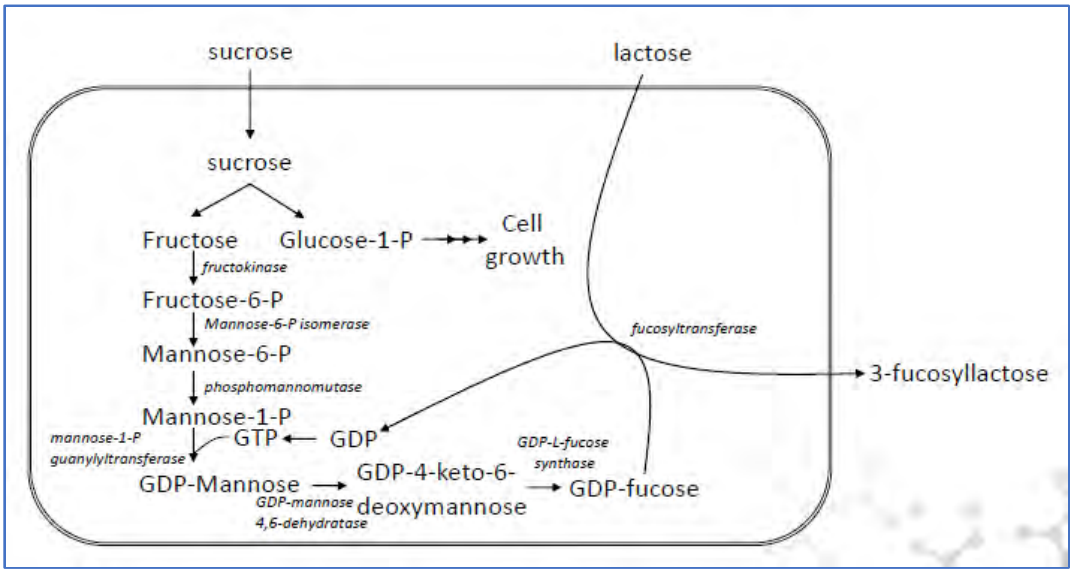



**Figure 4: 3FL Biosynthesis Pathway in *E. coli* K12 MG1655**

The strain was adapted to enable growth on sucrose, which is used to generate biomass as well as fructose. As shown in Figure 4, sucrose is used as a starting molecule in the production of GDP-fucose. To enable growth on sucrose and to generate fructose, two heterologous genes, EcCscB and BaSP, were inserted. EcCscB encodes the enzyme sucrose permease that facilitates intracellular uptake of sucrose while BaSP encodes sucrose phosphorylase that splits sucrose into glucose-1-phosphate and fructose. The glucose-1-phosphate is used by endogenous genes to generate biomass. The fructose is converted to fructose-6-phosphate by another enzyme, fructokinase, encoded by the heterologous gene ZmFrk, and subsequently to GDP-fucose.

To facilitate efficient intracellular 3FL production, over-expression of gene EcLacY ensures improved uptake of lactose for binding with the fucose moiety present in GDP-fucose. The GDP-fucose transfer step is enabled by the heterologous 3FT gene. Over-expression of endogenous EcMdfA encodes for a membrane protein which enables the transfer of intracellular 3FL into the extracellular environment, where it is subjected to downstream processing to yield the commercial 3FL product.

Genes encoding for unwanted proteins, including those for enzymes that interfere with the desired 3FL metabolic pathway, were deleted. These deletions help to make the strain more robust in industrial production settings, improve strain stability, and increase 3FL production.

*E. coli* K12 MG1655 INB008971 was characterized using whole genome sequencing and is deposited in DuPont's Global Culture Collection. The production strain is stable over 70 generations of fermentation. Stability of the strain was assessed using DNA sequencing data and by reviewing the consistency of repeated cell performance index values (i.e., 3FL titers in cultivated culture supernatant normalized to biomass production). DNA sequencing of the plasmid isolated from samples taken at the end of two fed batch fermentations showed that the plasmids are stable during the regular fermentation time.

**Host strain:** *E. coli* K12 MG1655

*E. coli* K12 MG1655 is derived from the well-known *E. coli* K12 strain via several classical mutagenesis steps and is available from both the American Type Culture Collection (ATCC) as ATCC 700926 and the Coli Genetic Stock Center (CGSC) as CGSC#7740. *E. coli* K12 MG1655 is classified by ATCC as a Biosafety Level 1 microorganism; the complete genome of this strain has been sequenced (GenBank Entry U00096.3).

*E. coli* K12 MG1655 also serves as the host strain in the production of DuPont's 2' fucosyllactose; GRAS notice GRN000749, incorporated by reference, included a discussion of the safety and characterization of *E. coli* K12 MG1655 on pp. 15-16.

**Synthetic Donor Genes:** DNA coding fragments with well characterized functions in the five strains listed were synthesized *in vitro* for insertion into the host strain. *In vitro* synthesis of inserted genes eliminated the potential introduction of undesirable genes from donor organisms.

| ***Name*** | ***Nature Identical Origin*** | ***Length (as bp)*** | ***Function*** | ***Location*** |
|---|---|---|---|---|
| 3FT | *Basilea psittacipulmonis* | 990 | α-1,3-Fucosyltransferase | Vector |
| BaSP | *Bifidobacterium adolescentis* | 1515 | Sucrose phosphorylase | Chromosome |
| ZmFrk | *Zymomonas mobilis* | 906 | Fructokinase | Chromosome |
| EcCscB | *Escherichia coli W* | 1248 | Sucrose transporter | Chromosome |
| EcLacY | *Escherichia coli K12* MG1655 | 1254 | Lactose permease | Chromosome |
| EcMdfA | *Escherichia coli K12* MG1655 | 1233 | MDR transporter | Chromosome |

**Production strain construction:** The general method used to introduce modifications into the host genome is shown in Figure 5 and was based on the work of Datsenko & Wanner (2000) and Snoeck et al. (2019).

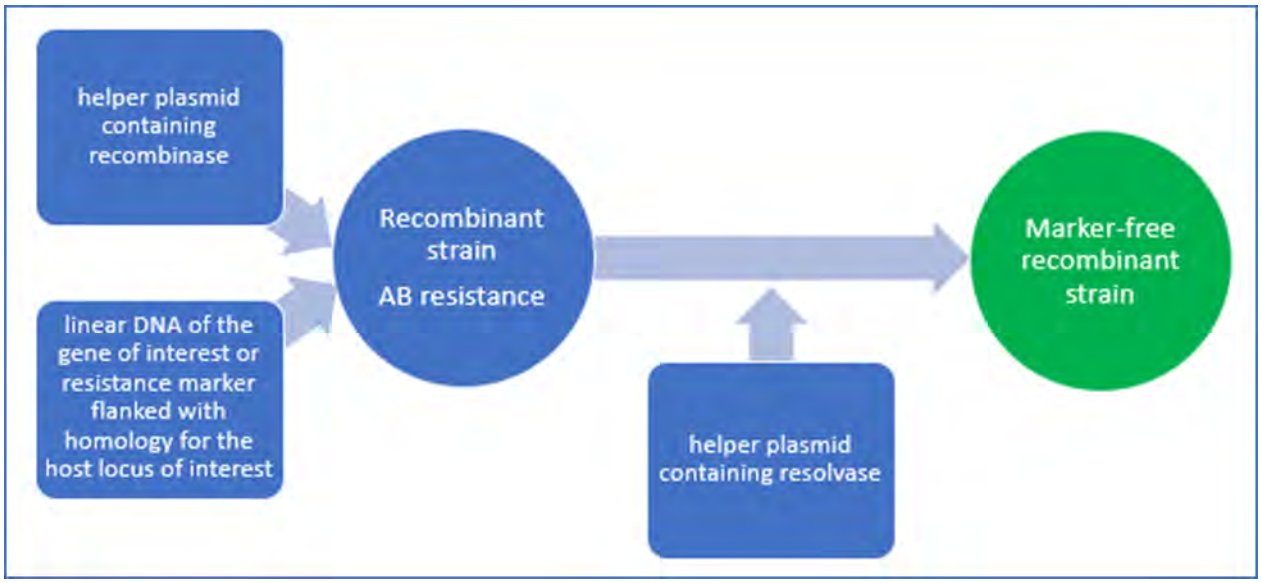



**Figure 5: Schematic of General Method for Introducing Modifications into the Host Genome**

To obtain a suitable production strain, *E. coli* K12 MG1655 was modified by engineering disruptions in genes that interfere with the metabolic pathway required to produce 3FL. The following deletions were made to remove the ability of the *E. coli* K12 MG1655 to produce the indicated proteins:

lacA (thiogalactoside acetyltransferase)
lacY (lactose permease)
lacZ (beta-D-galactosidase)
lon A (protease, enzyme with global regulatory function)
fsaA (fructose-6-phosphate aldolase 1)
agp (glucose-1-phosphatase/inositol phosphatase)
adhE (CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase/ pyruvate-formate-lyase deactivase)
ldhA (fermentative NAD-dependent D-lactate dehydrogenase)
pfkB (6-phosphofructokinase II)
wcaJ (putative UDP-glucose lipid carrier transferase)
setB (sugar efflux transporter)
thyA (thymidylate synthase)
glgC (glucose-1-phosphate adenylyltransferase)
pfkA (6-phosphofructokinase I)
fsaB (fructose-6-phosphate aldolase 2)
iclR (regulator in central intermediary metabolism, glyoxylate bypass)
pgi (glucose-6-phosphate isomerase)
arcA (negative response regulator of genes in aerobic pathways, with sensors ArcB and CpxA)
yhcE (partial deletion), yhcG & yhcF (putative proteins)
between wza (putative polysaccharide export protein) and yegH (putative transport protein) - full deletion
between flhA (flagellar biosynthesis protein) and yecH (uncharacterized DUF-containing protein - full deletion

To facilitate the production of GDP-fucose to support 3FL production, single copies of the following five genes were inserted in the *E. coli* K12 MG1655 genome. Each gene consisted of codon use-adapted coding sequences plus artificial promoters and terminators used to drive expression of the inserted coding sequences; these are described in De Mey et al. (2007).

BaSP (*B. adolescentis* sucrose phosphorylase gene)
ZmFrk (*Z. mobilis* fructokinase gene)
EcCscB (*E. coli* W anion symport for sucrose gene)
EcMdfA (*E. coli* K12 MG1655 MDR transporter gene)
EcLacY (*E. coli* K12 MG1655 lactose permease)

The modified *E. coli* K12 MG1655 was transformed into the production strain *E. coli* K12 MG1655 INB008971 through introduction of a plasmid vector containing the *B. psittacipulmonis* α-1,3-fucosyltransferase gene (3FT). Synthetized *de novo*, the plasmid vector has a pINB003937 backbone with a pBR322 type ORI; it lacks any conjugation, mobilization, or transfer functions and does not contain an antibiotic resistance marker. In addition to the 3FT gene, it contains homologous *E. coli* thyA as a selectable marker. The 3FT gene is controlled by an artificial promoter.

Some small and widely dispersed genetic scars were left in the *E. coli* K12 MG1655 genome after constructing the gene knockouts and gene insertions. However, no trace remains of the helper plasmid or the antibiotic marker used in the construction of the helper plasmid. Removal of the helper plasmid was validated by PCR and replica plating on a plate containing the antibiotic corresponding to the marker present on the helper plasmid.

**Production strain stability:** *E. coli* K12 MG1655 INB008971 is stable; as shown in Figures 6 and 7, no changes were observed in growth rate, production efficiency, and genetic make-up of the strain through 70 generations in a serial flask cultivation experiment.

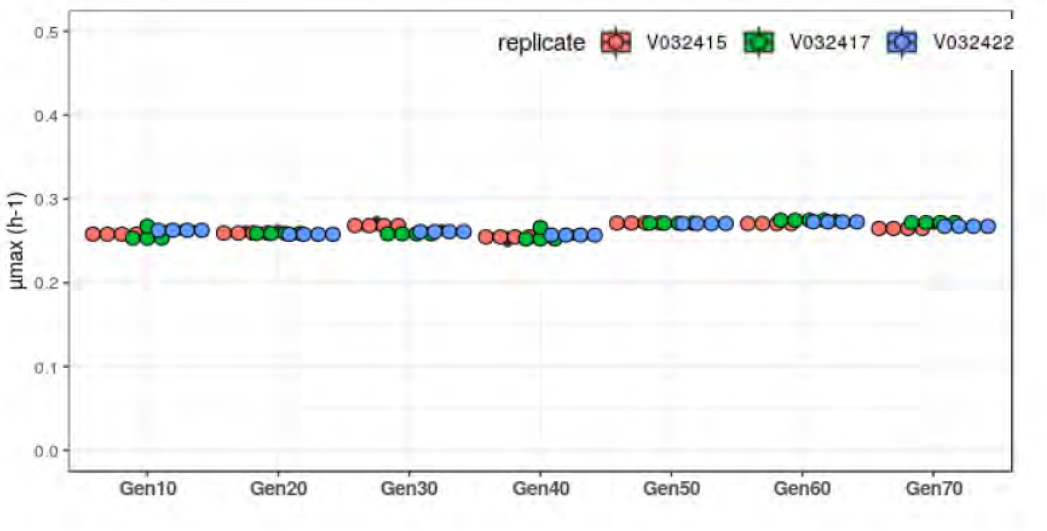



**Figure 6: Growth Rate Assessment of Three Vials over 70 Generations**

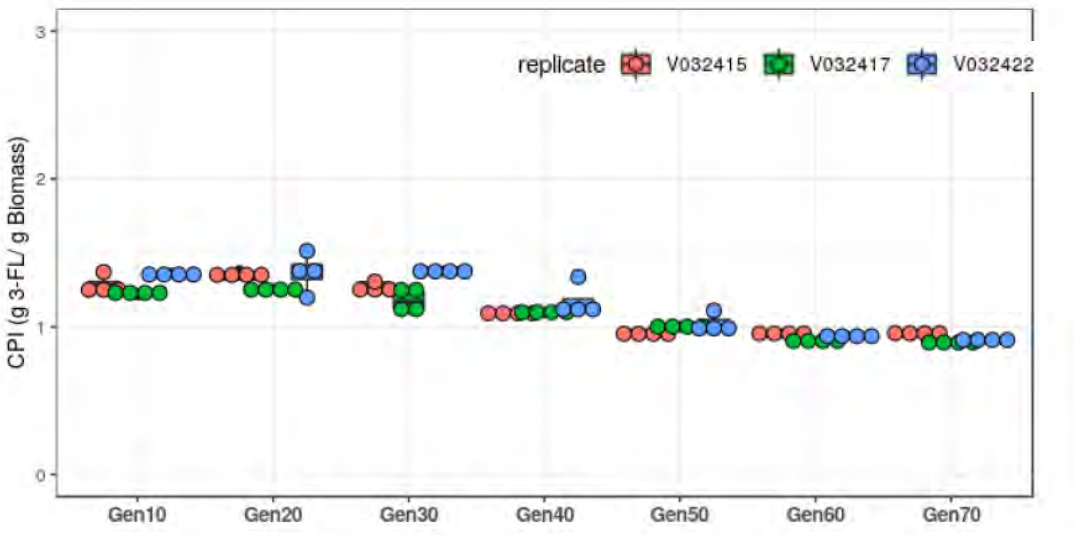



**Figure 7: 3FL Production Assessment of Three Vials over 70 Generations**

A comparison of next-generation sequencing (NGS) of the cultures and plasmid samples collected at the beginning and end (Gen 70) of the serial flask experiment detected no variants; the sINB008971 genome and the plasmid pINB003937 sequences were used as reference standards.

**Production strain safety:** No differences in pathogenicity are expected between the host strain and production strain, *E. coli* K12 MG1655 INB008971, as *E. coli* K-12 MG1655 is classified by ATCC as a Biosafety Level 1 microbial culture and the introduced genes are well characterized.

The absence of virulence factors unique to the production strain was verified by examining the whole genome sequencing (WGS) data of sINB008971 for the presence of genes coding for virulence factors using the Virulence Factors of Pathogenic Bacteria (VFDB) database and Virulence Finder analyzer tool. Virulence factors in the host strain, *E. coli* K-12 MG1655, were identified using the VFDB database and the Center for Genomic Epidemiology (CGE) Datasets. All virulence factors identified in the production strain were also present in the host strain.

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.

Bioinformatic analyses were carried out to assess the expressed proteins for potential allergenic cross-reactivity risk. The Access Food Allergy Research and Resource Program (FARRP) database (Version 19, released February 10, 2019) was searched using the 80 amino acid sliding window and the 8 contiguous identical amino acid (FAO/WHO 2001) methods. No matches were found.

The toxicity of the expressed proteins was evaluated by their homology with known protein toxins. Swiss-Prot, a manually annotated and reviewed section of the UniProt Knowledgebase (UniProtKB) database, was used as the primary data source for scientific literature on toxicity. The toxin homology was performed with BLASTP® command line using the Entrez protein query "toxin" in

the source database. Apart from the flagging of the endogenous genes EcMdfA and EcLacY, no other matches were found. As EcMdfA and EcLacY are also present in the host strain, their presence does not raise safety concerns.

Intrinsic antimicrobial resistance to MLS (macrolide, lincosamide, and streptogramin B) is encoded by the EcMdfA gene inherent in *E. coli* K12. The *E. coli* K12 MG1655 INB008971 genome contains one extra copy of the endogenous MdfA gene, which serves as an efflux transporter of 3FL, allowing its removal from the intact cell. Analysis of the *E. coli* K12 MG1655 INB008971 genome against bioinformatic databases for antibiotic/antimicrobial resistance genes (ResFinder and CARD) revealed no additional resistance compared to *E. coli* K12 and host strains.

### 2.3.3. Specifications and Batch Analysis of 3FL

The product specifications for 3FL and analytical data for four batches of 3FL are shown in Tables 1, 2, 3, and 4, below. With the exception of the carbohydrate analysis and the appearance parameters, DuPont has utilized standard methods for analysis of 3FL. The HILIC method used for carbohydrate analysis has been validated for this specific application. The consistency of the batch data shows that the product is manufactured reproducibly and conforms to specifications.

**Table 1. Appearance**

| Parameters | Specification | Method | Analyzed Batches | | | |
|---|---|---|---|---|---|---|
| | | | B90140 | B90157 | B90195 | B90196 |
| Appearance (Color) | White to ivory-colored | Visual | Pass | Pass | Pass | Pass |
| Appearance (Form) | Spray-dried powder | Visual | Pass | Pass | Pass | Pass |
| Appearance in solution | Clear, colorless to slightly yellow | Visual | Pass | Pass | Pass | Pass |

**Table 2. Carbohydrate Profile (HILIC method)**

| Parameters | Specification (Area under curve) | Analyzed Batches | | | |
|---|---|---|---|---|---|
| | | B90140 | B90157 | B90195 | B90196 |
| 3-Fucosyllactose | Min. 90% | 97.2 | 95.0 | 97.0 | 96.3 |
| Lactose | Max. 5% | < 0.2 | 2.6 | < 0.2 | 0.4 |
| Fucose | Max. 3% | 1.0 | 0.8 | 0.8 | 0.9 |
| Galactose/Glucose | Max. 3% | 1.5 | 1.3 | 1.4 | 1.4 |
| Other carbohydrates* | Max. 3% | 0.4 | 0.3 | 0.8 | 1.0 |

*Note: Calculated by difference i.e. Sum of all peaks (100%) minus 3FL, lactose, fucose, and galactose/glucose

**Table 3. Other Product Parameters & Impurities**

| Parameters | Specification | Method | Analyzed Batches | | | |
|---|---|---|---|---|---|---|
| | | | B90140 | B90157 | B90195 | B90196 |
| Water content | ≤ 5.0% | Karl Fischer titration | 4.4 | 3.88 | 4.39 | 3.8 |
| Protein content | ≤ 100 µg/g | Nanoquant (modified Bradford) | < 25 | < 25 | < 25 | < 25 |
| Total Ash | ≤ 0.5% | NMKL 173:2005, mod | < 0.12 | < 0.12 | < 0.12 | < 0.12 |
| Arsenic | ≤ 0.2 mg/kg | EN 15763:2010 | < 0.1 | < 0.1 | < 0.1 | < 0.1 |
| Cadmium | ≤ 0.05 mg/kg | EN 15763:2010 | < 0.01 | < 0.01 | < 0.01 | < 0.01 |
| Lead | ≤ 0.05 mg/kg | EN 15763:2010 | < 0.02 | < 0.02 | 0.04 (± 0.02) | < 0.02 |
| Mercury | ≤ 0.1 mg/kg | EN 15763:2010 | < 0.005 | < 0.005 | < 0.005 | < 0.005 |
| Endotoxins | ≤ 300 EU/g | Ph. Eur. 2.6.14 + Interference study | 53 | < 5.0 | 19 | < 5.0 |
| GMO detection (production strain rDNA) | Negative | PCR (internally validated; EFSA 2018) | Negative | Negative | Negative | Negative |

**Table 4. Microbiological Specifications**

| Parameters | Specification | Method | Analyzed Batches | | | |
|---|---|---|---|---|---|---|
| | | | B90140 | B90157 | B90195 | B90196 |
| Standard Plate Count | ≤ 1000 cfu/g | ISO 4833-1 | < 10 | < 10 | < 10 | < 10 |
| Yeast | ≤ 100 cfu/g | NMKL 98 | < 10 | < 10 | < 10 | < 10 |
| Mold | ≤ 100 cfu/g | NMKL 98 | < 10 | < 10 | < 10 | < 10 |
| Coliform/ Enterobacteriaceae | Absent in 10 g | ISO 21528-1 | Absent | Absent | Absent | Absent |
| *Salmonella* | Absent in 100 g | NMKL 71 | Absent | Absent | Absent | Absent |
| *Cronobacter sakazakii* | Absent in 100 g | ISO/TS 22964 | Absent | Absent | Absent | Absent |
| *Listeria monocytogenes* | Absent in 25 g | BRD 07/04-09/98 | Absent | Absent | Absent | Absent |
| *Bacillus cereus* | ≤ 10 cfu/g | NMKL 67-M | < 10 | < 10 | < 10 | < 10 |

### 2.3.4. Other Relevant Data

#### 1. Biogenic amines

The potential to produce biogenic amines was evaluated using screening methods for amino acids and biogenic amines. None of the tested batches contained any of the 20 common amino acids or biogenic amines, namely phenylethylamine, spermidine, spermine, histamine, putrescine, cadaverine, tryptamine, or tyramine.

#### 2. Allergens

None of the genes introduced into the production strain secreted proteins. Bioinformatic 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.

Batch data and other analyses demonstrate that DuPont's 3FL 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.

# PART 3. DIETARY EXPOSURE

## 3.1. Current Dietary Exposure

There is no public information on the current dietary exposures of 3FL by children, teenagers and adults. As 3FL is currently not being added to infant formulas and toddler foods in the US, there is also no public information on the current dietary exposure resulting from addition of 3FL.

## 3.2. Estimated Daily Intake for Proposed Uses

For non-exempt term infant formula and formulas intended for your children 12 months of age and older, the maximum intended use level is 0.2 g/serving (2.0 g/L) as prepared.

3FL is also proposed for use in other foods and beverages with maximum proposed use levels ranging from 0.14 to 4.0 g/serving. The estimated daily intake (EDI) of 3FL was derived based on food consumption records collected in the *What We Eat in America* (WWEIA) dietary component of the *National Health and Nutrition Examination Survey* (NHANES) 2013-2016. The NHANES is a continuous survey that uses a complex multistage probability sample designed to be representative of the civilian U.S. population. The NHANES datasets provide nationally representative nutrition and health data and prevalence estimates for nutrition and health status measures in the U.S. Statistical weights are provided by the National Center for Health Statistics (NCHS) to adjust for the differential probabilities of selection and non-response.

As part of the examination, trained dietary interviewers collected detailed information on all foods and beverages consumed by respondents in the previous 24-hour time period (midnight to midnight). A second dietary recall was administered by telephone three to ten days after the first dietary interview, but not on the same day of the week as the first interview. The dietary component of the survey is conducted as a partnership between the U.S. Department of Agriculture (USDA) and the U.S. Department of Health and Human Services (DHHS). DHHS is responsible for the sample design and data collection, and USDA is responsible for the survey's dietary data collection methodology, maintenance of the databases used to code and process the data, and data review and processing.

Using the NHANES 2013-2014 consumption data, Exponent Inc. estimated the 2-day average daily intake on a *per user* basis. *Per user* estimates refer to those who reported consuming any of the select foods included in the analysis on either of the survey days. The analysis was limited to individuals who provided two complete and reliable dietary recalls as determined by NCHS. The 2-day average intakes by each individual were estimated using Exponent's Foods Analysis and Residue Evaluation Program (FARE® version 13.04) software.

Table 5 lists the proposed food applications for 3FL addition, their corresponding maximum use levels, and examples of representative foods in the NHANES 2013-2016 database for each food category.

**Table 5: Proposed Food Applications and Use Levels of 3-FL**

| Food Category | Food Uses | 3FL Maximum Proposed Use Level (g/serving) | 3FL Maximum Proposed Use Level (g/kg) | Representative Foods in NHANES |
|---|---|---|---|---|
| Formula for infants and young children | Infant formula (0-12 months) | 0.2 | 2.0 | Infant formulas, ready-to-feed, prepared from powder/concentrate |
| | Formula targeted to young children aged 1-3 years | 0.2 | 2.0 | Toddler formulas, ready-to-feed, prepared from powder or concentrate |
| Foods for infants | Cereal & grain products, dry RTE (ready-to-eat) cereals, cookies, teething biscuit & toasts | 0.14 | 20 | Dry cereals (e.g., barley, mixed, oatmeal, rice, brown rice, multigrain), snack and sweets (e.g., cereal bars, cookies and teething cookies, crackers, puffs, crunchy snacks) |
| Foods for young children | Cereal & grain products, dry RTE (ready-to-eat) cereals, cookies, teething biscuit & toasts | 0.4 | 20 | Dry cereals (e.g., barley, mixed, oatmeal, rice, brown rice, multigrain), snack and sweets (e.g., cereal bars, cookies and teething cookies, crackers, puffs, crunchy snacks) |
| Juice and drinks for infants and young children | Juice/drinks | 0.2 | 2.0 | Juices including apple, mixed fruit, orange, pear, and grape juices |
| Baked goods & baking mixes | Cereal & nutrition bars | 1.2 | 30 | Cereal bars (e.g., Nutri-Grain bars, milk 'n cereal bar, granola bars) and nutrition bars (e.g., meal replacement bars, Cliff Bar, PowerBar, Slim Fast Bar, Zone Perfect Bar) |
| Beverages & beverage bases, nonalcoholic | Enhanced or fortified water | 0.3 | 1.2 | Enhanced or fortified waters (e.g., Propel Water, Glaceau Vitamin Water, SoBe Life Water) |
| | Energy, sports & isotonic drinks & mixes | 0.3 | 1.2 | Regular and low-calorie sport drinks (e.g., Gatorade, Powerade) and energy drinks (Full Throttle, Monster, NOS, Red Bull, Rockstar) |
| Breakfast cereals | Hot cereals | 1.6 | 31 | Oatmeal, cream of rice, cream of wheat, cream of rye, whole wheat hot cereal, oat bran hot cereal, grits, cornmeal mush |
| Breakfast cereals | RTE cereals | 1.6 | 40 | All types of RTE cereals |

| Food Category | Food Uses | 3FL Maximum Proposed Use Level (g/serving) | 3FL Maximum Proposed Use Level (g/kg) | Representative Foods in NHANES |
|---|---|---|---|---|
| Dairy product analogs | Milk substitutes, fluid | 0.3 | 1.2 | Soy milk, almond milk, rice milk, coconut milk (excluding coconut milk/cream used for cooking) |
| | Non-dairy yogurts | 2.0 | 12 | Soy and coconut milk yogurt |
| Milk products | Fermented milk, RTD (ready-to-drink) & mixes | 0.3 | 1.2 | Buttermilk and kefir |
| | Flavored Milk, RTD & mixes (including dairy-based beverages) | 0.3 | 1.2 | Flavored milk (e.g., chocolate and strawberry flavors), hot chocolate, milk shakes, malted milk drink |
| | Meal replacement beverages | 1.2 | 5 | Meal replacement beverages such as Carnation Instant Breakfast, Muscle Milk, Slim Fast, and high protein drinks |
| | Smoothies (dairy and non-dairy) | 1.2 | 5 | Fruit and/or vegetables smoothies (dairy and non-dairy types) |
| | Yogurt | 1.2 | 12 | Regular and Greek yogurt, all flavors, excluding frozen yogurt |
| Processed fruits and fruit juices | Fruit juices and nectars (including fruit-based beverages) | 0.3 | 1.2 | 100% fruit juices (excluding lemon juice), fruit juice drinks, fruit and vegetable juice drinks, nectars, and coconut water |
| Processed vegetables and vegetable juices | Vegetable juice | 0.3 | 1.2 | 100% vegetable juices |
| Foods for Special Dietary Use | Special dietary purpose ingredient in oral and enteral tube feeding ($\geq$ 11 years) | 4.0 | 20 | Not applicable |

Per-capita and per-user mean and 90$^{th}$ percentile results for the U.S. population and selected sub-populations in g/day and g/kg-bw/day are provided in Tables 6 and 7.

**Table 6: Per Capita Total 3FL Intake from All Proposed Uses by the Total U.S. Population and Subpopulations**

| Per Capita Population/ Subpopulation | N | % Users | Mean | | 90th Percentile | |
|---|---|---|---|---|---|---|
| | | | g/day | g/kg bw/day | g/day | g/kg bw/day |
| Total US Population | 12130 | 81 | 1.7 | 0.03 | 4.5 | 0.08 |
| Infants 0-6 mo | 259 | 72 | 1.1 | 0.18 | 2.3 | 0.38 |
| Infants 7-12 mo | 294 | 97 | 1.7 | 0.19 | 3.1 | 0.34 |
| Toddlers 13-35 mo | 673 | 96 | 1.7 | 0.14 | 4.3 | 0.37 |
| Children 3-12 y | 2611 | 96 | 1.6 | 0.06 | 3.4 | 0.14 |
| Adolescents 13-18 y | 1421 | 87 | 1.5 | 0.02 | 3.4 | 0.06 |
| Adults 19-49 y | 3537 | 77 | 1.7 | 0.02 | 4.5 | 0.06 |
| Adults 50+ y | 3335 | 77 | 1.9 | 0.02 | 5.1 | 0.07 |

**Table 7: Per User Total 3FL Intake from All Proposed Uses by the Total U.S. Population and Subpopulations**

| Per User Population/ Subpopulation | N | % Users | Mean | | 90th Percentile | |
|---|---|---|---|---|---|---|
| | | | g/day | g/kg bw/day | g/day | g/kg bw/day |
| Total US Population | 12130 | 81 | 2.2 | 0.04 | 5.0 | 0.10 |
| Infants 0-6 mo | 259 | 72 | 1.5 | 0.24 | 2.5 | 0.40 |
| Infants 7-12 mo | 294 | 97 | 1.7 | 0.19 | 3.1 | 0.35 |
| Toddlers 13-35 mo | 673 | 96 | 1.8 | 0.15 | 4.4 | 0.38 |
| Children 3-12 y | 2611 | 96 | 1.6 | 0.06 | 3.5 | 0.14 |
| Adolescents 13-18 y | 1421 | 87 | 1.7 | 0.03 | 3.9 | 0.06 |
| Adults 19-49 y | 3537 | 77 | 2.2 | 0.03 | 5.2 | 0.07 |
| Adults 50+ y | 3335 | 77 | 2.5 | 0.03 | 5.9 | 0.08 |

81% of the U.S. population were identified as consumers of foods wherein addition of 3FL is proposed. The mean and 90th percentile per-user EDIs for 3FL for the total U.S. population were 2.2 g/day (0.04 g/kg bw/day) and 5.0 g/day (0.10 g/kg bw/day), respectively. Per-user mean intakes of 3FL from all proposed food uses range from 1.5 g/day among infants aged 0-6 months to 2.5 g/day among older adults. On a bodyweight basis, the highest 3FL per-user mean intake was for infants age 0-6 months at 0.24 g/kg bw/day.

Infants, especially the 0-6 months age group, are expected to consume either breast milk or formula. As such, no increase in overall 3FL dietary exposure is anticipated by adding 3FL to infant formula.

As a special dietary purpose ingredient in oral and enteral tube feeding (> 11 years), 3FL is intended to be added at levels not exceeding 4 g/serving. These specially formulated foods are intended to be consumed only as necessary, under the guidance and direction of a physician, by the targeted patient population (≥ 11 years) to address particular dietary needs. Up to 3 servings of these foods may be consumed daily. Therefore, the maximum anticipated daily intake from foods for the specified special dietary use is 12 g/person/day, equivalent to 0.21 g/kg bw/day for a 56-kg adolescent and 0.16 g/kg bw/day for a 75-kg adult. No increase in overall 3FL dietary exposure in conventional food is anticipated by the incorporation of 3FL as an ingredient in foods for the specified special dietary use.

Tarleton et al. (2013) observe that the addition of low-digestible carbohydrates (CHO) to enteral formulas is intended to normalize bowel function and improve feeding tolerance, but suggests that the presence of certain comorbidities (bowel ischemia or severe dysmotility) may contra-indicate such addition. These are both easily observable conditions, and it is likely that the health professional overseeing the administration of partial or total enteral nutrition would be aware of the patient's status. Further, there are many randomized clinical trials and open-label studies in which low-digestible carbohydrates were added to enteral feedings given to preterm infants, children, healthy adults, bed-ridden elderly adults, and patients hospitalized for a variety of serious medical conditions. The test articles include partially hydrolyzed guar gum (PHGG), galactomannan, fructooligosaccharides (from scFOS to long-chain inulin), galactooligosaccharides, and GOS/FOS blends, with ingestion levels often greater than 20 g/day and as high as 63 g/day. No adverse effects were reported in any study, suggesting that addition of not more than 4 g of 3FL per serving of enteral feeds is safe.

A possibility exists that manufacturers of infant formula, enteral feeding solutions, and conventional foods may choose to use 3FL in conjunction with other low-digestible carbohydrates. We expect infant formula manufacturers to use our 3FL either alone within the level specified in this GRAS notice or in conjunction with other commercially manufactured human milk oligosaccharides within the levels of total oligosaccharides found in human milk, which is inherently well tolerated. Manufacturers might also use our ingredient in conjunction with other indigestible carbohydrates within ranges already established as well tolerated as per clinical trials.

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

For other uses, for which target levels cannot be established based on natural occurrence, appropriate studies of tolerance may be recommended. Here it must be recognized that it is not in a food or enteral formula manufacturer's interest to market a product that causes gastro-intestinal intolerance symptoms such as bloating or flatulence or more serious adverse reactions such as diarrhea or constipation. Thus, it is unlikely that a manufacturer planning a total nondigestible carbohydrate level in excess of the levels of 3FL contemplated in this GRAS notice would proceed without first obtaining data to support the safety and tolerability of the target level.

## 3.3. Estimated Dietary Exposure of Other Substances

### 3.3.1. Carbohydrates Other than 3FL

The commercial specifications for 3FL allow for the presence of small amounts of lactose (≤ 5%), fucose (≤ 3%), galactose/glucose (≤ 3%) and other carbohydrates (≤ 3%). Assuming each of the sugars/other carbohydrates are present at their maximum level in the commercial product, the anticipated mean per-user intake by infants (0-6 months) would be a maximum of 0.075 g/day for lactose, 0.045 g/day each for fucose, galactose/glucose, and other carbohydrates as a group.

Most commercial formulas manufactured in the United States contain lactose (72-74 g/L) from cow's milk as the sole source of carbohydrate (Raiten et al., 1998). The lactose is broken down into glucose and galactose prior to intestinal absorption. By comparison, the contribution of lactose, glucose, and galactose from the commercial 3FL product to the overall intake of these sugars from formula is insignificant.

Fucose is naturally present in breast milk in free form at mean concentrations of 20-30 mg/L (Choi et al., 2015). Therefore, when consuming the average amount of breast milk (840 ml/day[1]) for their age group, infants aged 0-6 months would consume a mean concentration of 0.02-0.04 g/day of free fucose. This value is comparable to the conservative anticipated mean per user intake of fucose (0.045 g/day) from the commercial 3FL product.

### 3.3.2. Microbial Endotoxins

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 endotoxins 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 analytical data from multiple batches (Part 2, Section 2.3.3) provides assurance that the 3FL product complies with the endotoxin specification.

### 3.3.3. 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 residues of the production microorganism in the 3FL product is demonstrated by microbial testing for coliform and *Enterobacteriaceae* using internationally recognized methods (ISO 21528-1) and by residual bacterial rDNA analysis by quantitative PCR (qPCR) to confirm the absence of production organism DNA following EFSA guidance (EFSA 2018) on evaluation of

[1] American Academy of Pediatrics: "General Guidelines for Baby Feeding" and "Amount and Schedule of Formula Feedings."

fermentation derived products for the absence of production strain and presence of DNA from the production strain.

As noted in the product data reported in Part 2, Section 2.3.3, all four batches tested negative for recombinant DNA from the GMM production host. The Limit of Detection (LoD) for the PCR reactions was below 10 ng DNA/g 3FL product, consistent with the DNA detection threshold set forward in the EFSA guidelines.

## PART 4. SELF-LIMITING LEVELS OF USE

The intended use of DuPont's 3FL is not self-limiting.

## PART 5. EXPERIENCE BASED UPON COMMON USE IN FOOD BEFORE 1958

The conclusion that the intended use of 3FL is GRAS is based on scientific procedures rather than experience based on common use in food prior to 1958.

# PART 6. NARRATIVE

The safety of 3FL and the information and data providing the basis for the conclusion that 3FL is GRAS for the intended conditions of use outlined in this dossier (Part 1, Section 1.3 and Part 3, Section 3.2) are discussed below.

## 6.1. Manufacturing and Purity of 3FL

3FL is a highly purified material, as established by the product specifications and documented by the batch analytical data. The primary components in the product are carbohydrates that historically present no cause for safety concerns. Post-fermentation down-stream processing steps effectively remove production host biomass and any associated rDNA, along with other impurities and contaminants. Screening of the 3FL production batches for bioamines, heavy metals, rDNA, endotoxins, and microbiological contaminants found these possible hazards to be either absent or within established product specifications (Part 2, Sections 2.3 and 2.4.)

The 3FL molecule, which makes up a minimum of 90% of the commercial 3FL product, is chemically and structurally identical to the 3FL molecule present in human breast milk. (See Part 2, Section 2.1.) The other minor carbohydrates, including lactose, fucose, glucose, and galactose, are normal components of the diet and are readily metabolized.

As noted in Part 3, Section 3.3.1., based upon the most conservative case where the maximum concentrations of these minor carbohydrates are present in the commercial 3FL product, the increase to the overall intake of 0.075 g/day lactose and 0.045 g/day each for glucose and galactose in cow's-milk-based infant formula is insignificant.

For fucose, the projected conservative intake of 0.045 g/day is comparable to mean per-user intakes (0.02-0.04 g/day) of breastfed infants aged 0-6 months consuming an average of 840 ml/day of breast milk.[1]

## 6.2. Safety of the Production Strain

*E. coli* K12 MG1655 INB008971 is a stable non-pathogenic and non-toxigenic strain with a safety profile comparable to its host, *E. coli* K12 MG1655, an ATCC Biosafety Level 1 microbial culture.

As noted in Part 2, Section 2.2., although bioinformatics analysis of sINB008971 WGS data found gene sequences coding for virulence factors and flagged the endogenous genes, EcMdfA and EcLacY, as toxic proteins, these genes are also present in *E. coli* K12 MG1655. Consequently, their presence in the production strain does not trigger a change in the biosafety level.

The introduced genes in the production strain express proteins facilitating 3FL production. None of these proteins is secreted into the fermenter. No gene sequences coding for allergenic proteins were identified when the sINB008971 WGS data examined against the FARRP database. Furthermore, no gene sequences coding for signal peptides were found, indicating that no protein excretion into the extracellular space occurred.

[1] American Academy of Pediatrics: "General Guidelines for Baby Feeding" and Amount and Schedule of Formula Feedings."

*E. coli* K12 MG1655 INB008971 contains one additional copy each of the EcMdfA and EcLacY genes. Insertion of the extra copy of these genes results in increases in the concentration of lactose permease and the MDR transport protein in the production strain cell membrane. (Both lactose permease and the MDR transport protein are naturally present in the host cell membrane.) However, these changes do not present a safety concern as both expressed proteins are removed during post-fermentation downstream processing. Several factors contribute to this certainty. First, both proteins are membrane bound and insoluble in water. Second, the molecular masses of lactose permease (~45 KDa) and MDR transport protein (~44 KDa) are individually 100-fold larger than 3FL (~0.5 KDa). And third, the microfiltration and/or ultrafiltration membranes used for this step of the process are specifically selected to remove cell biomass and large molecules (including proteins and endotoxins) from the 3FL product stream.

## 6.3. Experience Based on Common Use in Food

First isolated from human breast milk in the mid-1950s (Kunz 2012), 3FL belongs to a group of complex carbohydrates described as human milk oligosaccharides (HMOs) that are highly abundant in breast milk, representing approximately 20% of the total carbohydrate content of human milk (Urashima et al. 2012).

3FL concentrations in breast milk of healthy women vary across geographical regions, ethnicity, and lactation stage. Significantly higher average 3FL concentrations were reported by Thurl et al. (2010) in breast milk from women with Lewis blood group who lack the gene to secrete 2'-Fucosyllactose (2'FL). The biological reason for this variability in HMO concentrations is unknown.

A study reported by Erney et al. (2000), surveying 3FL concentrations in breast milk samples collected from 435 healthy women residing in 10 different countries, found that mean 3FL concentrations in breast milk samples collected from US women increased through the postpartum period, from a low of 1.03 g/L at 3-10 days, to 1.48 g/L at 11-30 days, reaching a high of 2.57 g/L at 31-217 days. The mean postpartum concentration was 1.84 g/L. Similar increases were observed in other regions: after 31 days, mean 3FL concentrations reached values of 2.15, 1.36, and 0.88 g/L in Asia, Europe, and Latin America, respectively.

Smilowitz et al. (2013) evaluated the compositional profile of breast milk collected at day 90 postpartum from 52 healthy US women and reported 3FL concentration levels ranging from 480 to 5600 μmol/L, with a mean of 2100 $\pm$ 1200 μmol/L (equivalent to 0.23 to 2.73 g/L and 1.03 $\pm$ 0.58 g/L), respectively.

Other studies reporting 3FL concentrations in breast milk collected from healthy women living other regions of the world include the following:

| Reference | Milk donor description | No. of samples | Days postpartum | 3FL concentration (g/L) | |
|---|---|---|---|---|---|
| | | | | Mean | Range |
| Austin et al. 2016 | 540 mothers living in urban areas of China | 446 | 5–11<br>12–30<br>31-60<br>61-120<br>>120 | 0.50<br>0.59<br>0.74<br>1.13<br>1.34 | 0.02[a] - 2.88<br>0.02[a] - 2.47<br>0.02[a] - 2.58<br>0.02[a] - 3.40<br>0.06 - 6.07 |
| Gabrielli et al. 2011 | 18 mothers with $Se^-/Le^+$ genotype, living in Italy, who delivered preterm newborns (mean gestational age: 27.9 weeks) | 72 | 4<br>10<br>20<br>30 | 1.92<br>2.05<br>1.34<br>2.22 | |
| Reference | Milk donor description | No. of samples | Days postpartum | 3FL concentration (g/L) | |
| Sumiyoshi et al. 2003 | 16 Japanese women | 46 | 4<br>10<br>30<br>100 | 0.23<br>0.28<br>0.43<br>0.45 | 0.01 - 1.01<br>0.04 – 0.77<br>0.02 – 1.42<br>ND – 1.49 |

[a] Samples having concentrations >LoD, but <LoQ (43 mg/kg) were assigned a value of half the LoQ (22 mg/kg); Specific gravity of breast milk = 1.03 kg/L

## 6.4. Safety Studies with 3FL

*In vitro*, *in vivo*, and animal feeding studies were conducted to supplement the data collected for the safety assessment and to support planned clinical and efficacy studies (Pitt et al. 2019). Studies conducted included acute oral toxicity and a 90-day subchronic rodent feeding study using test material conforming to the product specifications, and *in vitro* and *in vivo* assessments of genetic toxicity. The weight of evidence from these studies supports the safe use of 3FL produced using biotechnology as an ingredient in foods.

The acute oral toxicity study using female Crl:CD®(SD) rats as test animals was conducted in accordance with appropriate testing guidelines (US EPA, 2002; US FDA, 2007; OECD, 2008). A limit dose of 5000 mg/kg bw administered orally was selected based on the history of consumption and lack of toxicity historically observed with HMOs. No deaths or clinical signs of toxicity were observed over the 14-day observation period and there were no macroscopic observations at necropsy.

The subchronic rodent feeding study was conducted essentially as a limit-test in accordance with OECD test guideline 408 (OECD, 1998) and FDA Redbook guidance (US FDA, 2007), as there was no expectation of adverse effects, even at very high mg/kg bw intakes. However, the study design included two concentration levels of test substance rather than a single maximum concentration, which is typical for a limit-dose test, to provide an appropriate balance between animal welfare,

regulatory expectations, and business needs. Consequently, male and female Crl:CD®(SD) rats (n = 10 rats/sex/group) were fed either the basal diet or diets containing 5% 3FL, 10% 3FL, or 10% FOS (w/w), *ad libitum* for at least 90 consecutive days. Subchronic exposure of the rats to 3FL at 5% and 10% dietary concentrations did not produce any statistically significant or biologically-relevant differences in growth, feed intake, or feed efficiency ratio. There were no adverse clinical observations, and no clinical or anatomic pathology changes were observed. The average daily intakes of 3FL were equivalent to 5.98 and 7.27 g/kg bw/day for males and females, respectively.

The genetic toxicity potential of 3FL was evaluated *in vitro* using the bacterial reverse mutation (Ames) test, the mammalian cell micronucleus test in Chinese hamster ovary cells, and the chromosomal aberration test in human lymphocytes. 3FL was also evaluated in mice using the mammalian erythrocyte micronucleus test. There was no evidence of genetic toxicity in the bacterial reverse mutation test and chromosomal aberration assay. While there was a repeatable statistically-significant trend in the 4-h S9-activated test conditions in the *in vitro* micronucleus assay, the confirmatory *in vivo* mouse micronucleus study was negative at all doses.

### 6.4.1. Absorption, Distribution, Metabolism and Excretion (ADME)

3FL and other neutral HMOs are not hydrolyzed to the constituent monomers in the upper gastrointestinal tract; rather, most of the 3FL consumed passes through the gastrointestinal tract undigested. A low level of absorption does occur; the presence of intact 3FL has been reported in the plasma and excreted in the urine of healthy breastfed infants. Systemic exposure resulting from 3FL supplementation of infant formula or food is consistent with levels resulting from breast milk consumption.

Goehring et al. (2014) analyzed urine and blood samples of 17 healthy formula-fed and 16 healthy breastfed infants; multiple small molecular weight HMOs were detected in the urine and plasma of breastfed infants. Levels of 2FL, 3FL, and lacto-N-neotetraose (LNnT) in both plasma and urine were positively correlated with corresponding concentrations in breast milk. Relative to absorption of 2'FL and 6'-Sialyllactose (6'SL), representatives of major fucosyllated and acidic glycans present in human milk, were low; 0.1% of milk levels for plasma and 4% of milk levels for urine. This study confirms low levels of systemic exposure to 3FL and other HMOs and is consistent with other studies reporting the presence of HMOs in urine samples of breastfed infants.

Pharmacokinetic data from the 90-day rat feeding study described above (Pitt et al. 2019) provides an indication of systemic exposure to 3FL in 3FL-supplemented diets. Evaluation of blood and urine samples from rats fed a 5% or 10% 3FL-supplemented diet for 12-weeks indicated low absorption and systemic exposure from dietary intake that was proportional to dose. Recovery of 3FL in urine was ~0.4% (as mol %) of the administered dose in both dose groups. Thus, while measurable 3FL concentrations in the serum and urine confirmed low level absorption (well below 1.0% of the daily dietary intake levels), systemic exposure is negligible.

An *in-vitro* digestion study (Engfer et al. 2000) to assess the extent to which selected HMOs are hydrolyzed by enzymes present in the gastrointestinal tract found that neutral HMOs are very resistant to hydrolysis by secreted human pancreatic glycosidases and the enzymes bound to porcine or human intestinal brush border membranes of the upper small intestine. The studied neutral HMOs, including a total fraction of neutral HMOs, 2'FL, 3FL, and other non-fucosyllated

neutral HMOs, were recovered intact after the digestion step. Further analysis by MALDI-MS and enzymatic assays found no evidence of hydrolysis breakdown by-products.

Chaturvedi et al. (2001) investigated the fate of major neutral HMOs during transit through the gastrointestinal tract. The similarity between the HMO profile in breast milk and the urinary and fecal oligosaccharide profiles led to the conclusion that breast milk was the major source of oligosaccharides found in the urine and feces of breastfed infants. Oligosaccharide concentrations in urine samples were 1% of that of breast milk and while concentrations in fecal samples were about 10-fold higher than in breast milk. The investigators suggested that the findings indicate that approximately 97% of the consumed neutral HMOs, including 3FL and 2'FL, pass intact into the feces, while approximately 0.5% are absorbed and excreted into the urine.

### 6.4.2. 3FL Consumption in Human Milk vs. Dietary Exposure from Intended Use

The estimated dietary exposure to 3FL by infants (0-6 months), the highest consumers on a body weight basis, is comparable to the 3FL concentrations breastfed infants consume through breast milk.

Published studies in the literature report 3FL concentrations ranging from non-detectable to as high as 5.9 g/kg (Austin et al. 2016), equivalent to 6.08 g/L and 0.82 g/kg bw/day, based upon an average consumption of 840 ml breast milk per day and a body weight of 6.25 kg for infants aged 0-6 months.

Similowitz et al. (2013) reported 3FL concentrations of 0.23-2.73 g/L among women residing in the US, while Erney et al. (2000) found that 3FL mean concentrations increased over the postpartum period, reaching a high of 2.57 g/L at 31-217 days. These values are equivalent to a range of 0.03-0.37 g/kg bw/day with mean = 0.35 g/kg bw/day.

As the highest consumers of 3FL on a body weight basis, infants (0-6 months) represent the most sensitive population. As noted in Part 3, Section 3.2, the mean per user intake for infants (0-6 months) was 0.24 g/kg bw/day for the proposed applications and use levels. As breast milk is recommended as a sole source of nutrition for infants of this age group, breastfed infants are unlikely to consume the formula and other foods containing the added 3FL. As such, the overall dietary exposure for infants (0-6 months) is expected to be equal to the estimated mean daily intake from the proposed uses i.e. 0.24 g/kg bw/day and is below the mean 3FL daily intake levels (0.35 g/kg bw/day) resulting from consumption of breast milk.

## 6.5. Safety Assessment and GRAS Conclusion

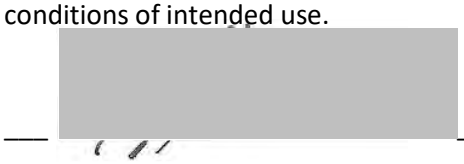
DuPont considers the following elements as key to our finding that 3FL is safe for use in the proposed food applications at the levels specified in Part 3, Section 3.2, and for reaching a GRAS conclusion under the conditions of intended use.

- 3FL is produced in accordance with good manufacturing practice in a food grade facility using food grade raw materials. It is a purified to yield a minimum 3FL assay of 90%; the 3FL molecule is chemically and structurally identical to that isolated from human breast milk. As evidenced by the batch data, the product can be manufactured reproducibly and consistently meets the specifications established to ensure product purity and safety.

- The production strain *E. coli* K12 MG1655 INB008971 is a stable non-pathogenic and non-toxigenic strain with a safety profile comparable to its host *E. coli* K12 MG1655, an ATCC Biosafety Level 1 microbial culture. The inserted genes are well characterized and synthetized *in-vitro* to avoid the potential introduction of undesirable genes from donor organisms.
- The in-vitro, in-vivo and animal feeding studies helps confirm that the biotechnologically produced 3FL product does not contain any impurities that would cause an adverse reaction when consumed at high levels.
- Absorption of 3FL is minimal; most of the ingested 3FL will be eliminated in the urine and feces. Systemic exposure resulting from 3FL added to infant formula and other food and beverages is expected to be very low and comparable with that in breastfed infants.
- At 0.24 g/kg bw/day, the mean (user only) dietary exposure levels of 3FL in the most sensitive subpopulation, Infants (0-6 months) is well within the 3FL intake range of breastfed infants (0.35 g/kg bw/day).
- The safety and ADME studies as well as the database and information to determine the dietary exposure and estimated daily intake for the proposed uses and by breastfed infants are publicly available.

## 6.6. Affirmative Statement Concerning Data & Information

I have reviewed the available data and information and am not aware of any data or information that are, or may appear to be, inconsistent with DuPont's conclusion of GRAS status under the conditions of intended use.



## PART 7. LIST OF SUPPORTING DATA AND INFORMATION


Andreas NJ, Kampmann B, Mehring Le-Doare K. 2015. Human breast milk: A review on its composition and bioactivity. *Early Human Dev* 9111:629-635.

Austin S, De Castro CA, Benet T, Hou Y, Sun H, Thakkar SK, Vinyes-Pares G, Zhang Y, Wang P. 2016. Temporal change of the content of 10 oligosaccharides in the milk of Chinese urban mothers. *Nutrients* 8:346.

American Academy of Pediatrics. 2020. *Amount and schedule of formula feedings* https://www.healthychildren.org/English/ages-stages/baby/formula-feeding/Pages/Amount-and-Schedule-of-Formula-Feedings.aspx

American Academy of Pediatrics. 2020. *General guidelines for baby feeding* https://www.healthychildren.org/English/ages-stages/baby/feeding-nutrition/Pages/How-Often-and-How-Much-Should-Your-Baby-Eat.aspx

Chaturvedi P, Warren CD, Buescher CR, Pickering LK, Newburg DS. 2001. Survival of human milk oligosaccharides in the intestine of infants. *Adv Exp Med Biol* 501:315-323.

Choi SS, Lynch BS, Baldwin N, Dakoulas EW, Roy S, Moore C, Thorsrud BA, Röhrig CH. 2015. Safety evaluation of the human-identical milk monosaccharide, L-fucose. *Regul Toxicol Pharmacol* 721.:39-48.

Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 9712:6640-6645.

De Mey M, Maertens J, Lequeux GJ, Soetaert WK, Vandamme EJ.. 2007. Construction and model-based analysis of a promoter library for *E. coli*: An indispensable tool for metabolic engineering. *BMC Biotechnol* 7:34.

EFSA 2018. Guidance on the characterization of microorganisms used as feed additives or production organisms; Section 3.2 – evaluation of fermentation products for presence of DNA from the production strain. *EFSA Journal*. 163:5206.

Engfer MB, Stahl B, Finke B, Sawatzki G, Daniel H. 2000. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. *Am J Clin Nutr* 71:1589-1596.

Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman–Leyer KM, O'Ryan ML, Ruiz–Palacios G, Hilty MD, Pickering LK, Prieto PA. 2000. Variability of human milk neutral oligosaccharides in a diverse population. *J Pediatr Gastroenterol Nutr* 30:181-192.

FAO/WHO 2001. *Evaluation of allergenicity of genetically modified foods*. Report of a Joint FAO/WHO expert consultation on allergenicity of foods derived from biotechnology, Rome, Italy, 21–25 January: 1-15.

Gabrielli O, Zampini L, Galeazzi T, Padella L, Santoro L, Peila C, Giuliani F, Bertino E, Fabris C, Coppa GV. 2011. Preterm milk oligosaccharides during the first month of lactation. *Pediatrics* 1286:e1520-e1531.

Goehring KC, Kennedy AD, Prieto PA, Buck RH. 2014. Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. *PLoS ONE* 9:e101692.

Kunz C. 2012. Historical aspects of human milk oligosaccharides. *Adv Nutr* 3:30S-39S.

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

Raiten DJ, Talbot JM, Waters JH. 1998. *Assessment of nutrient requirements for infant formulas*: LSRO report prepared for the Center for Food Safety and Applied Nutrition, Food and Drug Administration, Department of Health and Human Services, Washington, DC 20204 under contract no. 223-92-2185. Bethesda, MD: American Institute of Nutrition.

Smilowitz JT, O'Sullivan A, Barile D, German JB, Lonnerdal B, Slupsky CM. 2013. The human milk metabolome reveals diverse oligosaccharide profiles. *J Nutr* 143:1709-1718.

Snoeck N, De Mol ML, Van Herpe D, Goormans A, Maryns I, Coussement P, Peters G, Beauprez J, De Maeseneire SL, Soetaert W. 2019. Serine integrase recombinational engineering SIRE.: A versatile toolbox for genome editing. *Biotechnol Bioeng* 116:364-374.

Sumiyoshi W, Urashima T, Nakamura T, Arai I, Saito T, Tsumura N, Wang B, Brand-Miller J, Watanabe Y, Kimura K. 2003. Determination of each neutral oligosaccharide in the milk of Japanese women during the course of lactation. *Br J Nutr* 891:61-69.

Tarleton SM, Kraft CA, DiBaise JK. 2013. Fiber-enriched enteral formulae: advantageous or adding fuel to the fire? *Practical Gastroenterol*, December:11-22.

Thurl S, Munzert M, Henker J, Boehm G, Müller-Werner B, Jelinek J, Stahl B. 2010. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. *Br J Nutr* 104:1261-1271.

van Leeuwen SS, Schoemaker RJ, Gerwig GJ, van Leusen-van Kan EJ, Dijkhuizen L, Kamerling JP. 2014. Rapid milk group classification by 1H NMR analysis of Le and H epitopes in human milk oligosaccharide donor samples. *Glycobiology* 24:728-739.