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Safety of 2′-fucosyllactose (2′-FL) produced by a derivative strain (APC199) of *Corynebacterium glutamicum* ATCC 13032 as a novel food pursuant to Regulation (EU) 2015/2283

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Abstract

Following a request from the European Commission, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked to deliver an opinion on 2′-fucosyllactose (2′-FL) as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The NF is mainly composed of the human-identical milk oligosaccharide (HiMO) 2′-FL, but it also contains D-lactose, L-fucose, 3-fucosyllactose, difucosyllactose, D-glucose and D-galactose. The NF is produced by fermentation with a genetically modified strain (APC199) of *Corynebacterium glutamicum* ATCC 13032. 2′-FL, when chemically synthesised or produced by fermentation with derivative strains of *Escherichia coli* K-12 DH1 or *E. coli* BL21 (DE3), is already authorised and included in the EU list of NFs. This application refers to a change in the production process and specifications, while target population, conditions of use and consequently, the anticipated intake remain unchanged. The information provided on the identity, production process, composition and specifications of the NF does not raise safety concerns. The intake of other carbohydrate-type compounds structurally related to 2′-FL is also considered of no safety concern. In line with other milk oligosaccharides that are natural components of human milk, the safety assessment of this NF is mainly based on the comparison between the intake of breastfed infants and the estimated intake as NF. Given that the NF would be consumed at the same extent as the already authorised 2′-FL, the Panel considers that the consumption of the NF at the proposed uses and use levels does not raise safety concerns. The Panel concludes that the NF is safe under the proposed conditions of use.

Keywords: 2′-fucosyllactose, 2′-FL, human milk oligosaccharide, HMO, HiMO, novel food, safety

Requestor: European Commission

Question number: EFSA-Q-2020-00500

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

On 29 May 2020, the company Advanced Protein Technologies Corporation submitted a request to the Commission in accordance with Article 10 of Regulation (EU) 2015/2283[1] to change the specifications of the authorised novel food (NF) 2′-fucosyllactose (2′-FL) so as to include 2′-FL produced by *Corynebacterium glutamicum* (strain APC199).

2′-FL produced by either chemical synthesis or microbial fermentation using derivate strains of *Escherichia coli* K-12 DH1 or *E. coli* BL21 (DE3) is already authorised to be used in a number of foods, food for special medical purposes (FSMP) as defined in Regulation (EU) No 609/2013[2], and food supplements (FS) as defined in Directive 2002/46/EC[3].

The applicant has requested data protection according to the provisions of Article 26 of Regulation (EU) 2015/2283.

In accordance with Article 10(3) of Regulation (EU) 2015/2283, the European Commission (EC) asks the European Food Safety Authority (EFSA) to provide a scientific opinion on the proposed changes in the specifications of the NF 2′-FL.

1.2. Interpretation of the Terms of Reference

The present application seeks a change in the production process and specifications of the authorised 2′-FL, so as to include 2′-FL produced by a derivative strain (APC199) of *C. glutamicum* ATCC 13032, without proposing any changes in its conditions of use. 2′-FL is included in the Union list of authorised NFs (Commission Implementing Regulation (EU) 2017/2470[4]) when chemically synthesised (Commission Implementing Decision (EU) 2016/376[5]) or produced by fermentation with derivative strains of *E. coli* K-12 DH1 (Commission Implementing Regulation (EU) 2019/338[6]) or *E. coli* BL21 (DE3) (Commission Implementing Regulation (EU) 2017/2201[7]). Therefore, the current assessment exclusively focuses on the proposed changes with regards to the possible impact on the safety and nutritional aspects.

1.3. Additional information

2′-FL is included in the Union list of authorised NFs (Commission Implementing Regulation (EU) 2017/2470[4]) when produced by chemical synthesis (EFSA NDA Panel, 2015a) or fermentation with genetically modified strains of *E. coli* K-12 DH1 or *E. coli* BL21 (DE3). A 2′-FL/difucosyllactose (DFL) mixture produced by fermentation with a genetically modified strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019a) and 3-fucosyllactose (3-FL), a constitutional isomer of 2′-FL produced by fermentation with a derivative strain of *E. coli* K-12 MG1655 (EFSA NDA Panel, 2021), are also included in the Union list of authorised NFs. Moreover, the extension of use in FS for infants of 2′-FL and 2′-FL/DFL mixture, both produced with derivative strains of *E. coli* K-12 DH1, and the safety of 3-FL produced with a

[1] Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. OJ L 327, 11.12.2015, pp. 1–22.

[2] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009. OJ L 181, 29.6.2013, pp. 35–56.

[3] 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 food supplements. OJ L 183, 12.7.2002, pp. 51–57.

[4] Commission Implementing regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, pp. 72.

[5] Commission Implementing Decision (EU) 2016/376 of 11 March 2016 authorising the placing on the market of 2′-O-fucosyllactose as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council. OJ L 70, 16.3.2016, pp. 27–31.

[6] Commission Implementing regulation (EU) 2019/338 of 11 March 2019 authorising the change of the specifications of the novel food 2′-fucosyllactose produced with *Escherichia coli* K-12 under Regulation (EU) No 2015/2283 of the European Parliament and of the Council. OJ L 70, 12.3.2019, pp. 21–24.

[7] Commission Implementing regulation (EU) 2017/2201 of 27 November 2017 authorising the placing on the market of 2′-fucosyllactose produced with *Escherichia coli* strain BL21 as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council. OJ L 313, 29.11.2017, pp. 5–9.

derivative strain of *E. coli* BL21 (DE3), have recently been assessed by EFSA with positive outcomes (EFSA NDA Panel, 2022a,b,c).

Since 2015, several scientific opinions have been adopted by the EFSA NDA Panel on the safety of human-identical milk oligosaccharides (HiMOs) as NFs pursuant to Regulation (EC) No 258/97 or Regulation (EU) 2015/2283:

- Chemically synthesised 2′-FL (EFSA NDA Panel, 2015a);
- Chemically synthesised lacto-*N*-neotetraose (LNnT) (EFSA NDA Panel, 2015b) and LNnT produced with derivative strains of *E. coli* BL21 (DE3) (EFSA NDA Panel, 2020a);
- Extension of use in FS for infants of chemically synthesised 2′-FL and LNnT (EFSA NDA Panel, 2015c) or 2′-FL and LNnT produced with derivative strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2022a);
- Chemically synthesised *N*-acetyl-D-neuraminic acid (NANA) (EFSA NDA Panel, 2017);
- 2′-FL/DFL mixture produced with a derivative strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019a);
- Lacto-*N*-tetraose (LNT) produced with a derivative strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019b) or with derivative strains of *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022d);
- Extension of use in FS for infants of 2′-FL/DFL mixture and LNT produced with derivative strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2022b);
- 3-FL produced with a derivative strain of *E. coli* K-12 MG1655 (EFSA NDA Panel, 2021) or with a derivative strain of *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022c).
- 3′-Sialyllactose (3′-SL) sodium salt produced with a derivative strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020b) or with derivative strains of *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022e);
- 6′-Sialyllactose (6′-SL) sodium salt produced with a derivative strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020c).

2. Data and Methodologies

2.1. Data

The safety assessment of this NF is based on data supplied in the application, information submitted by the applicant following EFSA requests for supplementary information and additional data identified by the Panel.

Administrative and scientific requirements for NF applications referred to in Article 10 of Regulation (EU) 2015/2283 are listed in Commission Implementing Regulation (EU) 2017/2469[8].

A common and structured format on the presentation of NF applications is described in the EFSA guidance on the preparation and presentation of a NF application (EFSA NDA Panel, 2016). As indicated in this guidance, it is the duty of the applicant to provide all of the available (proprietary, confidential and published) scientific data, (including both data in favour and not in favour) that are pertinent to the safety of the NF.

This NF application includes a request for protection of proprietary data in accordance with Article 26 of Regulation (EU) 2015/2283. The data requested by the applicant to be protected comprise: (i) identity of the NF; (ii) toxicological information; (iii) information on the genetically modified production strain.

2.2. Methodologies

The assessment follows the methodology set out in the EFSA guidance on NF applications (EFSA NDA Panel, 2016) and the principles described in the relevant existing guidance documents from the EFSA Scientific Committee. The legal provisions for the assessment are laid down in Article 11 of Regulation (EU) 2015/2283 and in Article 7 of Commission Implementing Regulation (EU) 2017/2469. The legal provisions for the assessment of food intended for infants and young children, FSMP, and total diet replacement for weight control are laid down in Regulation (EU) No 609/2013 and,

[8] Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, pp. 64–71.

respectively, in Commission Delegated Regulation 2017/1798[9] (total diet replacement for weight control), in Commission Delegated Regulation (EU) 2016/128[10] (FSMP), and in Commission Delegated Regulation (EU) 2016/127[11] (as regards the specific compositional and information requirements for infant formula (IF) and follow-on formula (FOF) and as regards requirements on information relating to infant and young child feeding).

This assessment concerns only the risks that might be associated with consumption of the NF under the proposed conditions of use, and is not an assessment of the efficacy of the NF with regard to any claimed benefit. This assessment also is not an assessment on whether the NF is suitable as stipulated by Regulation (EU) No 609/2013.

3. Assessment

3.1. Introduction

The NF, which is the subject of the application, contains 2′-FL as primary constituent (≥ 94% w/w dry matter (DM)), a fucosylated neutral trisaccharide consisting of L-fucose linked via an α-(1–2′) bond to the D-galactose moiety of D-lactose. 2′-FL has been reported, on average, as the most abundant component within the complex fraction of oligosaccharides naturally occurring in human milk (HMO), in the general population of breastfeeding women (Erney et al., 2000). The concentration of 2′-FL in human milk depends on the lactation period, with higher levels reported in the colostrum (Thurl et al., 2017). The Panel notes that although 2′-FL is the major component of the NF, related substances, namely D-lactose, L-fucose, 3-FL, DFL, D-glucose and D-galactose are also present. The NF is produced by fermentation with *C. glutamicum* APC199, a derivative strain of *C. glutamicum* ATCC 13032.

2′-FL produced by chemical synthesis (EFSA NDA Panel, 2015a) or fermentation with derivative strains of *E. coli* K-12 DH1 or *E. coli* BL21 (DE3) has already been authorised as a NF in the EU (Commission Implementing Regulation 2017/2470). The 2′-FL/DFL mixture produced by fermentation with a genetically modified strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019a) and 3-FL, a constitutional isomer of 2′-FL produced by fermentation with a derivative strain of *E. coli* K-12 MG1655 (EFSA NDA Panel, 2021), are also included in the Union list of authorised NFs. Moreover, the extension of use in FS for infants of 2′-FL and the 2′-FL/DFL mixture, both produced with derivative strains of *E. coli* K-12 DH1, and the safety of 3-FL produced with a derivative strain of *E. coli* BL21 (DE3), have recently been assessed by EFSA with positive outcomes (EFSA NDA Panel, 2022a,b,c).

The applicant has not proposed any changes in the conditions of use, therefore, the intended uses and use levels of the NF remain the same as those already authorised for 2′-FL manufactured by chemical synthesis or fermentation with derivative strains of *E. coli* K-12 DH1 or BL21 (DE3) and included in the Union list of NFs.

The target population is the general population, except for the use of 2′-FL in FS, which is intended for individuals ≥ 1 year of age.

The applicant indicated that, according to Regulation (EU) 2015/2283, this NF falls under the following categories:

i) 'food with a new or intentionally modified molecular structure, where that structure was not used as, or in, a food within the Union before 15 May 1997'; and
ii) 'food consisting of, isolated from or produced from microorganisms, fungi or algae'.

3.2. Identity of the NF

The NF is a powdered mixture mainly composed of 2′-FL (≥ 94.0% w/w DM), but it also contains D-lactose (≤ 3.0% w/w DM), L-fucose (≤ 3.0% w/w DM), 3-FL (≤ 3.0% w/w DM), DFL (≤ 2.0% w/w DM), D-glucose (≤ 3.0% w/w DM) and D-galactose (≤ 3.0% w/w DM). It is produced by fermentation

[9] Commission Delegated Regulation (EU) 2017/1798 of 2 June 2017 supplementing Regulation (EU) No 609/2013 of the European Parliament and of the Council as regards the specific compositional and information requirements for total diet replacement for weight control. OJ L 259, 7.10.2017, pp. 2–10.

[10] Commission Delegated Regulation (EU) 2016/128 of 25 September 2015 supplementing Regulation (EU) No 609/2013 of the European Parliament and of the Council as regards the specific compositional and information requirements for food for special medical purposes. OJ L 25, 2.2.2016, pp. 30–43.

[11] Commission Delegated Regulation (EU) 2016/127 of 25 September 2015 supplementing Regulation (EU) No 609/2013 of the European Parliament and of the Council as regards the specific compositional and information requirements for infant formula and follow-on formula and as regards requirements on information relating to infant and young child feeding. OJ L 25, 2.2.2016, pp. 1–29.

with a genetically modified strain (APC199) of *C. glutamicum* ATCC 13032. 2′-FL is a trisaccharide consisting of L-fucose linked via an α-(1-2′) bond to the D-galactose moiety of D-lactose (Table 1 and Figure 1). 2′-FL is a constitutional isomer of 3-FL, which contains the same monosaccharide moieties as those present in 2′-FL but with an α-(1-3) bond between L-fucose and the D-glucose moiety of D-lactose.

Table 1: Chemical identity of 2′-FL

Chemical substance	
Chemical (IUPAC) name	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Common name	2′-Fucosyllactose
Synonyms and abbreviations	2′-Fucosyl-D-lactose; 2′-O-fucosyllactose; 2′-FL
CAS number	41263-94-9
Molecular formula	$C_{18}H_{32}O_{15}$
Molecular weight	488.44 Da

The molecular structure of 2′-FL was determined by liquid chromatography–tandem mass spectrometry (LC–MS/MS) based on the retention time, accurate mass and fragmentation pattern, which allowed to differentiate between 2′-FL α-(1″-2′) and 3-FL α-(1″-3), and by comparison with a high purity standard.

The identity of 2′-FL was also confirmed by high-performance anion-exchange chromatography–pulsed amperometric detection (HPAEC–PAD) by comparison with a high purity standard.

The molecular structure of 2′-FL was confirmed by mono-dimensional (1D) nuclear magnetic resonance (NMR) spectroscopy, including ^{1}H, ^{13}C, ^{13}C-DEPT-90 (distortionless enhancement by polarisation transfer) and ^{13}C-DEPT-135 spectra, and two-dimensional (2D) NMR spectroscopy, including ^{1}H-^{1}H COSY (correlated spectroscopy), TOCSY (total correlation spectroscopy), ^{1}H-^{13}C HSQC (heteronuclear single quantum coherence spectroscopy), HSQC-TOCSY, ^{1}H-^{13}C HMBC (heteronuclear multiple-bond coherence spectroscopy) and ROESY (rotating-frame nuclear Overhauser effect spectroscopy) spectra. The correlations demonstrating the α-(1″-2′) bond between L-fucose and the D-galactose moiety of D-lactose and the links between the three different pyranose rings, H-4/C-1′ (H-1′/C-4) and H-2′/C-1″ (H-1″/C-2′), were evidenced on the HMBC spectrum.

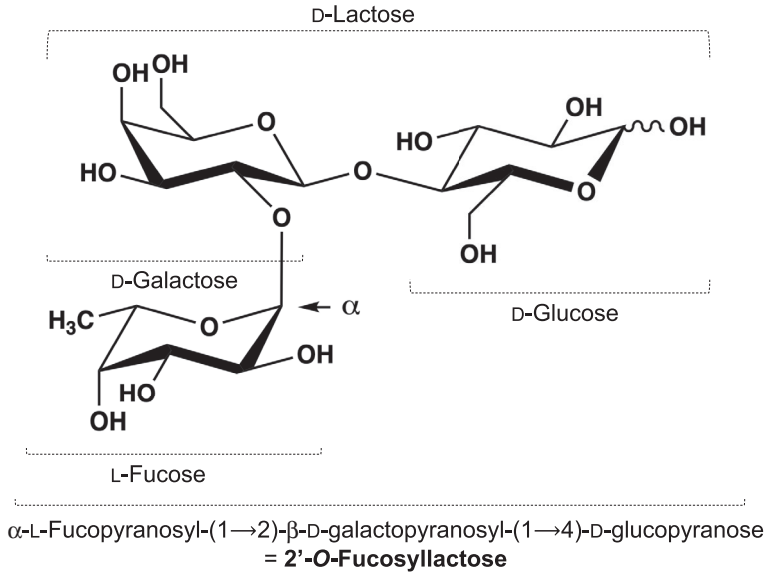

Figure 1: Chemical structure of 2′-FL

The 2′-FL produced by the microbial fermentation described has been shown to be chemically and structurally identical to authentic standards by LC–MS/MS, HPAEC-PAD and 1D and 2D NMR spectroscopy, and the Panel considers it as being a HiMO.

3.3. Production process

According to the information provided by the applicant, the NF is produced in line with Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Points (HACCP) principles, in a facility that is FSSC (Food Safety System Certification) 22000 certified.

The NF is produced by fermentation using a genetically modified strain derived from the host strain *C. glutamicum* ATCC 13032. The production strain *C. glutamicum* APC199 has been modified to effectively synthesise 2′-FL. Glucose and lactose are used as carbon source and substrate, respectively, by *C. glutamicum* APC199 to produce 2′-FL, which is released into the fermentation media. At the end of the fermentation process, the bacterial biomass is heat inactivated and removed by microfiltration. The isolation, purification and concentration of the product involve a series of filtration steps and drying to obtain purified 2′-FL in powder form.

The production strain *C. glutamicum* APC199 is a genetically modified derivative of the host strain *C. glutamicum* ATCC 13032. *C. glutamicum* has been recommended for the qualified presumption of safety (QPS) status but only for production purposes (EFSA Scientific Committee, 2007; EFSA BIOHAZ Panel, 2022), which implies the absence of viable cells of the production microorganism in the final product and can also be applied for food and feed products based on microbial biomass. The production strain has been deposited at the Korean Collection of Type Cultures (KCTC). A detailed description of the genetic modification steps applied to obtain the production strain has been provided by the applicant. No residual DNA from the production strain was detected in the NF by quantitative polymerase chain reaction (qPCR) amplification of 10 target genes, including 4 antimicrobial resistance genes either introduced during the genetic modification or identified in the whole genome sequence (WGS) analysis of the production strain, in accordance with the EFSA statement on WGS analysis (EFSA, 2021). The absence of both DNA and viable cells from the production strain has been demonstrated in accordance with the EFSA Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018).

The Panel considers that the production process is sufficiently described and does not raise safety concerns.

3.4. Compositional data

Batch-to-batch analyses showed that the NF consists of 2′-FL as the main component (98.26% w/w DM[12]). The remaining constituents[12] include D-lactose (< 0.14% w/w DM), L-fucose (< 0.07% w/w DM), 3-FL (< 0.32% w/w DM), DFL (< 0.28% w/w DM), D-glucose (< 0.28% w/w DM) and D-galactose (0.18% w/w DM).

The NF can be described as a white to off-white/ivory powder. The solubility in water was measured in five batches of the NF according to the Organisation for Economic Cooperation and Development (OECD) test guideline (TG) 105 (OECD, 1995), resulting in an average value of 716.6 g/L at 20°C.

In order to confirm that the manufacturing process is reproducible and adequate to produce on a commercial scale a product with certain characteristics, the applicant provided analytical information for five batches of the NF (Table 2). Information was provided on the accreditation of the laboratories that conducted the analyses presented in the application.

Table 2: Batch-to-batch analysis of the NF

Parameter	Batch number					Method of analysis[(a)]
	#1	#2	#3	#4	#5	
Composition						
2′-FL (% w/w DM)	97.47	98.26	98.04	98.67	98.85	HPAEC-PAD (validated internal method)[(b)]
D-Lactose (% w/w DM)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
L-Fucose (% w/w DM)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
3-FL (% w/w DM)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
DFL (% w/w DM)	< LOQ	0.35	< LOQ	< LOQ	< LOQ	

[12] Average content in five batches of the NF. For those batches of the NF where the levels of any carbohydrate by-product were below the respective limit of quantification (LOQ), the concentration of the corresponding compound has been considered to be equal to the respective LOQ value for the purpose of calculating its average content in the five batches of the NF.

Parameter	Batch number					Method of analysis[a]
	#1	#2	#3	#4	#5	
D-Glucose (% w/w DM)	0.27	0.34	0.31	< LOQ	< LOQ	
D-Galactose (% w/w DM)	0.21	0.22	0.22	0.17	0.08	
Water (%)	1.67	1.74	1.64	2.46	2.70	Karl Fischer titration
Ash (%)	0.17	0.15	0.14	0.03	0.09	AOAC 923.03 (gravimetry)
Protein (%)	< 0.0003	< 0.0003	0.0005	< 0.0003	< 0.0003	Bradford assay
Contaminants						
Arsenic (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	ISO 17294:2014 mod. (ICP-MS)
Cadmium (mg/kg)	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	
Lead (mg/kg)	< 0.005	0.010	< 0.005	< 0.005	0.011	
Mercury (mg/kg)	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	
Aflatoxin M1 (μg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	KMFDS general test method 9.2.3 (HPLC/FLD)
Ethanol (mg/kg)	221	220	221	229	232	USP 467 (GC/FID)
Microbial parameters						
Total plate count (CFU/g)	< 10	< 10	< 10	< 10	< 10	AOAC 990.12
Coliforms (CFU/g)	< 10	< 10	< 10	< 10	< 10	AOAC 991.14
Yeasts (CFU/g)	< 10	< 10	< 10	< 10	< 10	ISO 21527-2
Moulds (CFU/g)	< 10	< 10	< 10	< 10	< 10	ISO 21527-2
Enterobacteriaceae (in 10 g)	ND	ND	ND	ND	ND	ISO 21528-1
Salmonella spp. (in 25 g)	ND	ND	ND	ND	ND	ISO 6579-1
Cronobacter spp. (in 10 g)	ND	ND	ND	ND	ND	ISO/TS 22964 IDF/RM 210:2006
Staphylococcus aureus (CFU/g)	< 100	< 100	< 100	< 100	< 100	ISO 6888-1
Bacillus cereus (CFU/g)	< 100	< 100	< 100	< 100	< 100	US FDA BAM (Chapter 14)
Clostridium perfringens (in 25 g)	ND	ND	ND	ND	ND	AOAC 976.30
Listeria monocytogenes (in 25 g)	ND	ND	ND	ND	ND	US FDA BAM (Chapter 10)
Sulphite-reducing Clostridia spores (CFU/g)	< 10	< 10	< 10	< 10	< 10	BS ISO 15213-2003
Endotoxins (EU/g)	7.2	5.7	< 5.0	< 5.0	35.5	Ph. Eur. 2.6.14

2′-FL: 2′-Fucosyllactose; 3-FL: 3-Fucosyllactose; AOAC: Association of Official Analytical Collaboration; BS: British standard; CFU: Colony forming unit; DFL: Difucosyllactose; DM: Dry matter; EU: Endotoxin unit; GC/FID: Gas chromatography–flame ionisation detection; HPAEC-PAD: High-performance anion-exchange chromatography–pulsed amperometric detection; HPLC/FLD: High-performance liquid chromatography–fluorescence detection; ICP-MS: Inductively coupled plasma–mass spectrometry; IDF/RM: International Dairy Federation/Reviewed method; ISO: International Organization for Standardization; KMFDS: Korean Ministry of Food and Drug Safety; LOQ: Limit of quantification; mod.: Modification of sample preparation methods; ND: Not detected; Ph. Eur.: European Pharmacopeia; TS: Technical specification; US FDA BAM: US Food and Drug Administration – Bacteriological Analytical Manual; USP: United States Pharmacopeia.

(a): Analytical methods have been reported by the applicant as listed in the column or 'equivalent'.

(b): LOQs: 2′-FL = 1.19% w/w DM; D-Lactose = 0.14% w/w DM; L-Fucose = 0.07% w/w DM; 3-FL = 0.32% w/w DM; DFL = 0.26% w/w DM; D-Glucose = 0.24% w/w DM; D-Galactose = 0.07% w/w DM.

The Panel considers that the information provided on the composition is sufficient for characterising the NF.

3.4.1. Stability

Stability of the NF

The applicant carried out stability studies under normal (25°C and 60% relative humidity (RH)) and accelerated (40°C and 75% RH) storage conditions with five batches of the NF, three monitored up to 104 weeks and two additional ones, up to 81 weeks. The five batches were analysed for 2′-FL and moisture content. Microbiological parameters (standard plate count, yeast and mould, and *Salmonella*)

were also monitored under normal storage conditions for up to 104 weeks for three batches of the NF and up to 56 weeks, for the two additional ones.

No appreciable changes in 2′-FL and moisture content were observed up to 104 weeks under both normal and accelerated storage conditions. Microbial parameters were below the respective limits of detection over the 104-week normal storage period. The applicant proposed a 24-month shelf-life under ambient conditions for the NF.

The Panel considers that the data provided sufficient information with respect to the proposed stability of the NF for two years.

Stability of the NF under the intended conditions of use

The applicant carried out stability tests with one batch of the NF in IF stored at 4, 25 or 37°C for 18 months. No significant changes in the 2′-FL content were observed over the storage period, regardless of the temperature. The stability of five batches of the NF in corn silk tea was also investigated at 25°C and 60% RH for up to 81 weeks, with no noticeable changes in the concentration of 2′-FL. Moreover, 2′-FL has been demonstrated to be stable in various food matrices, including IF, yoghurt, ready-to-drink flavoured milk and citrus fruit beverages (EFSA NDA Panel, 2015a).

The Panel considers that the available information is sufficient with respect to the stability of the NF in the proposed food matrices.

3.5. Specifications

The specifications of the NF are indicated in Table 3.

Table 3: Specifications of the NF

Description: 2′-Fucosyllactose (2′-FL) is a white to off-white/ivory powder produced by microbial fermentation and further isolated and purified.	
Source: A genetically modified strain (APC199) of *Corynebacterium glutamicum* ATCC 13032	
Parameter	**Specification**
Composition	
2′-FL (% w/w DM)	≥ 94
D-Lactose (% w/w DM)	≤ 3
L-Fucose (% w/w DM)	≤ 3
3-FL (% w/w DM)	≤ 3
DFL (% w/w DM)	≤ 2
D-Glucose (% w/w DM)	≤ 3
D-Galactose (% w/w DM)	≤ 3
Water (%)	≤ 9.0
Ash (%)	≤ 0.5
Protein (%)	≤ 0.005
Contaminants	
Arsenic (mg/kg)	≤ 0.03
Aflatoxin M1 (μg/kg)	≤ 0.025
Ethanol (mg/kg)	≤ 1,000
Microbial parameters	
Total plate count (CFU/g)	≤ 500
Yeast and mould (CFU/g)	≤ 100
Enterobacteriaceae (in 10 g)	ND
Salmonella (in 25 g)	ND
Cronobacter spp. (in 10 g)	ND
Endotoxins (EU/g)	≤ 100

2′-FL: 2′-Fucosyllactose; 3-FL: 3-Fucosyllactose; CFU: Colony forming unit; DFL: Difucosyllactose; DM: Dry matter; EU: Endotoxin unit; ND: Not detected.

As compared to the specifications for the authorised 2′-FL produced with derivative strains of *E. coli* K-12 DH1 or *E. coli* BL21 (DE3), a few differences have been proposed for 2′-FL produced with a

derivative strain (APC199) of *C. glutamicum* ATCC 13032: higher purity (from ≥ 83% or ≥ 90% to ≥ 94% 2′-FL) and lower limits for certain carbohydrate by-products (D-lactose, 3-FL and DFL), while specifications for 2′-fucosyl-D-lactulose and fucosylgalactose (present in the authorised 2′-FL) are not included; lower specifications for protein (from ≤ 0.01% to ≤ 0.005%), arsenic (from ≤ 0.2 mg/kg to ≤ 0.03 mg/kg) and total plate counts (from ≤ 3,000 or ≤ 10,000 CFU/g to ≤ 500 CFU/g); and the inclusion of a maximum limit for ethanol.

The Panel considers that the information provided on the specifications of the NF is sufficient and does not raise safety concerns.

3.6. History of use of the NF and/or of its source

3.6.1. History of use of the NF

There is no history of use of the NF. However, 2′-FL, which is the major constituent of the NF, is already included in the Union list of NFs when manufactured by chemical synthesis or fermentation with derivative strains of *E. coli* K-12 DH1 or *E. coli* BL21 (DE3). It is authorised to be added to a variety of food categories (e.g. dairy products, beverages), including foods for special groups (e.g. IF and FOF) and FS, excluding FS for infants (intended for individuals above 1 year of age).

2′-FL is the most represented oligosaccharide in human milk with mean concentrations ranging from 2.1 to 2.8 g/L (Erney et al., 2001; Thurl et al., 2017; Soyyılmaz et al., 2021) and maximum means up to 4.28 or 4.78 g/L (Thurl et al., 2017; Soyyılmaz et al., 2021, respectively).

In bovine milk, oligosaccharides are 20 times less concentrated than in human milk and acidic oligosaccharides are the most abundant oligosaccharides (i.e. 6′-SL), while fucosylated ones (i.e. 2′-FL) are found at very small concentrations (Aldredge et al., 2013; Urashima et al., 2013).

3.7. Proposed uses and use levels and anticipated intake

The applicant does not intend to amend the uses and use levels already authorised for 2′-FL manufactured by chemical synthesis or fermentation with derivative strains of *E. coli* K-12 DH1 or *E. coli* BL21 (DE3) and included in the Union list of NFs. Therefore, the NF would be consumed at the same extent as the already authorised 2′-FL and no estimate of the intake has been carried out.

3.7.1. Precautions and restrictions of use

The same restrictions of use as those already authorised apply, i.e. excluding the use as FS in infants. FS are not intended to be used if other foods with added 2′-FL or human milk (for young children) are consumed on the same day.

3.8. Absorption, distribution, metabolism and excretion (ADME)

No ADME data have been provided for the NF.

As reported in previous EFSA opinions (EFSA NDA Panel, 2014, 2019a,b, 2020a,b, 2021, 2022c,d) HMOs, including fucosyllactoses, are considered 'non-digestible oligosaccharides' (EFSA NDA Panel, 2014) since they do not undergo any significant digestion in the upper gastrointestinal tract (Brand-Miller et al., 1995, 1998; Engfer et al., 2000; Gnoth et al., 2000, 2001; Chaturvedi et al., 2001; Rudloff and Kunz, 2012).

Brand-Miller et al. (1995, 1998) reported that HMOs, consumed as a load (a purified oligosaccharide fraction from human milk), are fermented in the colon by the intestinal microbiota. Chaturvedi et al. (2001) and Coppa et al. (2001) reported that 97% and 40–50%, respectively, of the ingested HMOs are excreted unchanged in faeces of breastfed infants. Furthermore, approximately 1–2% of the ingested amounts of HMOs is excreted unchanged in the infants' urine (Rudloff et al., 1996, 2006; Goehring et al., 2014; Kunz et al., 2017; Vazquez et al., 2017; EFSA NDA Panel, 2019b).

Based on information available on HMOs, the Panel considers that the NF does not undergo any significant digestion in the gastrointestinal tract and that only small amounts are expected to be absorbed. Moreover, there are no indications that the absorption of 2′-FL, which is the main constituent of the NF, or other structurally related mono- and oligosaccharides (e.g. D-lactose), differs from that of similar components found in human milk.

3.9. Nutritional information

The NF is mainly composed of the non-digestible oligosaccharide 2′-FL.

The NF contains other carbohydrates individually present at low concentrations. D-Lactose is the most abundant molecule in human milk (~ 7 g/100 mL) and its monomers, D-glucose and D-galactose, are normal constituents of human milk. L-Fucose is also found in human milk (Smilowitz et al., 2013) at concentrations ranging from 20 to 30 mg/L (Choi et al., 2015). DFL also belongs to the group of fucosylated HMOs, which constitute on average about 70% of the total HMO fraction in human milk (Bode, 2012). DFL concentration in human milk depends on the lactation period, with higher levels reported in colostrum (Coppa et al., 1999, 2001; Erney et al., 2000; Morrow et al., 2004; Musumeci et al., 2006; Asakuma et al., 2008, 2011; Thurl et al., 2010, 2017; Galeotti et al., 2012, 2014; Spevacek et al., 2015; Austin et al., 2016; McGuire et al., 2017). 3-FL is another fucosylated HMO and one of the most abundant neutral core HMOs, with increasing concentrations in human milk over the course of lactation (Erney et al., 2001; Thurl et al., 2017; Samuel et al., 2019).

The Panel considers that taking into account the composition of the NF and the proposed conditions of use, consumption of the NF is not nutritionally disadvantageous.

3.10. Toxicological information

The list of toxicological studies, which were provided and claimed proprietary by the applicant, is reported in Table 4. All main studies were conducted in accordance with OECD principles of Good Laboratory Practice (GLP) (OECD, 1998a) and TG 471 (OECD, 1997a), 473 (OECD, 1997b), 487 (OECD, 2016), 474 (OECD, 2014) and 408 (OECD, 1998b).

Table 4: List of toxicological studies with the NF

Reference	Type of study	Test system	Dose
Study No. B18674 (Unpublished, 2019a)	Bacterial reverse mutation test (GLP, OECD TG 471)	*Salmonella* Typhimurium TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101)	313, 625, 1,250, 2,500 and 5,000 μg/plate (absence and presence of S9 mix)
Study No. B18675 (Unpublished, 2019b)	*In vitro* mammalian chromosomal aberration test (GLP, OECD TG 473)	Chinese Hamster Lung (CHL/IU) cells	1,250–5,000 μg/mL (absence and presence of S9 mix)
Study No. 3267-292 (Unpublished, 2021)	*In vitro* human lymphocyte micronucleus test (GLP, OECD TG 487)	Blood from healthy donors	2,500–5,000 μg/mL (absence and presence of S9 mix)
Study No. B18676 (Unpublished, 2019c)	*In vivo* micronucleus test (GLP, OECD TG 474)	Mouse, CrlOri:CD1(ICR),	2,500, 5,000, 7,500 mg/kg (oral administration via gastric intubation twice at 24-h intervals)
Study No. B18672 (Unpublished, 2019d)	Single Oral Dose Toxicity Study (GLP, Notification No. 2017–71 of Korean Ministry of Food and Drug safety)	Sprague–Dawley Rats	2,500, 5,000 & 7,500 mg/kg (10 mL/kg bw. Oral administration by gavage; 14-d observation period after dosing)
Study No. B18673 (Unpublished, 2019e)	90-day repeated dose oral toxicity study with a 4-week recovery period (GLP, OECD TG 408 (1998b))	Sprague–Dawley Rats	2,500, 5,000 & 7,500 mg/kg (10 mL/kg bw. Oral administration by gavage)

GLP: Good Laboratory Practice; OECD: Organisation for Economic Co-operation and Development; bw: body weight.

3.10.1. Genotoxicity

The potential genotoxicity of the NF was investigated in a bacterial reverse mutation test, an *in vitro* mammalian cell micronucleus test, an *in vitro* chromosomal aberration test in human lymphocytes and also in an *in vivo* micronucleus test in mice (Table 4).

The *in vitro* assessment of the mutagenic potential of the NF (Unpublished Study Report, 2019a) was performed with mutants of *S.* Typhimurium, strains TA98, TA100, TA1535 and TA1537, and a mutant of *E. coli* WP2 uvrA (pKM101). A mutagenicity test was conducted with the plate incorporation

method at five different concentrations from 313 up to 5,000 μg/plate (main study), either in the presence or absence of liver microsomal fractions (S9 fraction) with the NF in water solution. No reproducible or dose-related increases in revertant colony numbers over control counts were observed with any of the strains following exposure to 2′-FL at any concentration. No appreciable toxicity or precipitation was observed following exposure to any dose of the NF.

In the *in vitro* mammalian cell micronucleus test in CHL cells (Unpublished Study Report, 2019b), after a dose range finding study, concentrations of 2′-FL of 1,250, 2,500 and 5,000 μg/mL (main study) were tested after 4 or 24 h exposure in the presence or absence of metabolic activation (S9 fraction). No toxicity to cells or precipitation have been observed and the percentage of micronuclei was not significantly increased in any of the test substance concentrations.

In addition, 2′-FL was also evaluated for its ability to induce micronuclei *in vivo* in the bone marrow of ICR mice (Unpublished Study Report, 2019c). Mice were treated orally with 2′-FL twice at a 24-h interval. Following a dose range finding study, doses of 2,500, 5,000 and 7,500 mg/kg body weight (bw) (main study) were tested in 5 male mice/dose. The incidence of micronucleated cells and the ratio of polychromatic erythrocytes to total erythrocytes in the NF treated groups was not statistically significantly different from the negative control group.

Finally, the applicant also provided the report of an *in vitro* human lymphocytes chromosomal aberration test (Unpublished Study Report, 2021). After evaluation of cytotoxicity and possible precipitation in a dose range finding experiment, the study was performed with the NF at concentrations of 2,500, 4,000 and 5,000 μg 2′-FL/mL in the presence (3-h) or absence (3- and 24-h exposure) of metabolic activation (S9 fraction). Only at the low concentration in the 24-h exposure, a statistically significant increase in chromosomal aberrations was noted, but the increase was still within the historical control values of the laboratory. No dose-dependent toxicity to cells or precipitation has been observed and the NF did not induce other statistically significant increases in the frequency of structural and numerical chromosomal aberrations in any of the three exposure conditions.

Taking into account the studies provided and considering the nature, source and production process of the NF, the Panel considers that there are no concerns regarding genotoxicity.

3.10.2. Acute and subacute toxicity

The applicant has provided an acute toxicity study in rats. The Panel considers that in general acute toxicity studies are not pertinent for the safety assessment of NFs.

3.10.3. Subchronic toxicity

The applicant provided a 90-day oral toxicity study where groups of 10 Crl:CD(SD) neonatal Sprague–Dawley rats/sex (7-day old at the start of treatment) were given daily doses of 2,500, 5,000 and 7,500 mg per kg bw of 2′-FL by oral gavage. Additional rats (5 sex/dose) for the control and high dose groups were observed over a 4-week recovery period. The Panel notes that, although the study was conducted in the last quarter of 2018 according to the relevant OECD guidance document (TG 408), the version of the guidance followed (1998) was not the most updated one (version of June 2018). Therefore, some endocrine and reproductive endpoints are not included (e.g. thyroid parameters, vaginal cytology).

One female of the high-dose group was found dead on day 30. Mortality was ascribed to an incorrect gavage (post-mortem examination), therefore considered as an accidental death. One male from the intermediate-dose group also was found dead on day 72. It was considered as an incidental death of the rat in absence of any findings at clinical and post-mortem examination, histopathology included. Statistically significant findings are indicated in Appendix A.

Episodes of soft stool and diarrhoea were frequently observed in all the rats of both sexes receiving the high dose, starting from the end of the first month of treatment. With the exception of the first day, these findings were not present in the subsequent 4-week recovery period. Some statistically significant changes were noted on body weight (limited to a decrease observed in high-dose males on day 4 (5.8%) and at the intermediate dose on day 11 (5.9%)), on food consumption (decrease in females at high dose on day 16, 13.6%) and on hindlimb grip strength (decrease in females at all dose levels at the end of the treatment period (15–16%)).

Statistically significant variations were also noted on laboratory parameters: haematological, biochemical and coagulation parameters (e.g. monocytes, creatinine, prothrombin time – details of all these variations are included in Appendix A) and decrease in urinary volume (37–44%). Regarding statistically significant changes in organ weights, increase in the relative liver weight in both male and

female rats (11.3–15.5%) at high dose, intermediate (9.1%, females) and low dose (13.1%, males) were recorded. Furthermore, a decrease in absolute brain weight (6.4%, females) and thymus weight (16.7%, males) at the end of the recovery period was also recorded. No gross or histopathological findings considered to be treatment related were noted. The changes observed were generally of low magnitude, temporary, without dose correlation or observed at the end of the recovery period or limited to only one sex and, overall, they are considered by the Panel as not biologically relevant.

The author of the study proposed the high dose level of 7,500 mg 2′-FL/kg bw as the no observed adverse effects level (NOAEL). However, the Panel notes that even in the absence of changes in body weight, food consumption and gross- or histological changes the highest dose tested induced soft stool and diarrhoea in almost all animals of both sexes in the second and third month of treatment. The Panel identified the intermediate dose of 5,000 mg 2′-FL/kg bw per day as the NOAEL.

3.10.4. Human data

No human intervention studies with the NF have been provided by the applicant and no reference to human data was made.

3.11. Allergenicity

The applicant did not identify any allergenic potential of introduced proteins as a result of the genetic modification of the *C. glutamicum* ATCC 13032 host strain according to the relevant opinion on the assessment of allergenicity (EFSA GMO Panel, 2010). The criterion used for identifying allergenic proteins was that of considering 'higher than 35% identity in a sliding window of 80 amino acids'.

The protein content in the NF is low (≤ 0.005%) as indicated in the specifications (Table 3).

The Panel considers that, for these reasons, the likelihood of allergenic reactions to the NF is low.

4. Discussion

The NF is a powdered mixture mainly composed of the HiMO 2′-FL, but it also contains D-lactose, L-fucose, 3-FL, DFL, D-glucose and D-galactose. The NF is produced by fermentation with *C. glutamicum* APC199, a derivative strain of *C. glutamicum* ATCC 13032.

This application is limited to a change in the production process and specifications. The information provided on the production process, composition, identity and specifications of the NF does not raise safety concerns. The applicant intends to add the NF to a variety of foods, including IF and FOF, FSMP and FS. However, the applicant does not intend to amend the uses and use levels already authorised for 2′-FL and included in the Union list of NFs. Therefore, since the NF produced with the new process has similar composition and would be consumed at the same extent as the already authorised 2′-FL, no estimate of the intake has been carried out.

The submitted genotoxicity studies did not raise safety concerns. Due to diarrhoea observed in the subchronic toxicity study at the highest dose tested (7,500 mg 2′-FL/kg bw), the Panel identified the intermediate dose of 5,000 mg 2′-FL/kg bw per day as the NOAEL in this study. In line with other milk oligosaccharides that are natural components of human milk, the safety assessment is mainly based on the comparison between the intake of breastfed infants and the estimated intake as NF.

Given the composition of the NF and that the NF would be consumed at the same extent as the already authorised 2′-FL, the Panel considers that the consumption of the NF is safe at the proposed uses and use levels.

5. Conclusions

The Panel concludes that the NF, which is composed of 2′-FL and other structurally related mono- and oligosaccharides, is safe under the proposed conditions of use.

5.1. Protection of proprietary data in accordance with Article 26 of Regulation (EU) 2015/2283

The Panel could not have reached the conclusion on the safety of the NF under the proposed conditions of use without the data claimed as proprietary by the applicant: (i) identity of the NF as confirmed by NMR spectroscopy; (ii) toxicological information including genotoxicity studies, and subchronic toxicity studies (Table 4); (iii) information on the genetically modified production strain (DNA analysis and absence of viable cells).

6. Steps taken by EFSA

1) On 13 October 2020, EFSA received a letter from the European Commission with the request for a scientific opinion on the safety of 2′-fucosyllactose (2′-FL). Ref. Ares(2020) 5447489.
2) On 13 October 2020, a valid application on 2′-FL, which was submitted by Advanced Protein Technologies Corporation, was made available to EFSA by the European Commission through the Commission e-submission portal (NF 2020/1825) and the scientific evaluation procedure was initiated.
3) On 15 February 2021, EFSA requested the applicant to provide additional information to accompany the application and the scientific evaluation was suspended.
4) On 26 May 2022, additional information was provided by the applicant through the Commission e-submission portal and the scientific evaluation was restarted.
5) On 21 July 2022, EFSA requested the applicant to provide additional information to accompany the application and the scientific evaluation was suspended.
6) On 15 September 2022, additional information was provided by the applicant through the Commission e-submission portal and the scientific evaluation was restarted.
7) During its meeting on 26 October 2022, the NDA Panel, having evaluated the data, adopted a scientific opinion on the safety of 2′-FL as a NF pursuant to Regulation (EU) 2015/2283.

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Abbreviations

1D	Mono-dimensional
2D	Two-dimensional
2′-FL	2′-Fucosyllactose
3-FL	3-Fucosyllactose
3′-SL	3′-Sialyllactose
6′-SL	6′-Sialyllactose
ADME	Absorption, Distribution, Metabolism and Excretion
A/G ratio	Albumin/Globulin ratio
ALT	Alanine aminotransferase
AOAC	Association of Official Analytical Collaboration
ATCC	American Type Culture Collection
BIOHAZ	EFSA Panel on Biological Hazards
BS	British standard
bw	Body weight
CAS	Chemical Abstracts Service
CFU	Colony forming unit
CHL/IU	Chinese Hamster Lung cells
COSY	Correlated spectroscopy
Crl:CD(SD) rats	Charles River Laboratories: Cesarean-derived (Sprague Dawley) rats
CrlOri:CD1(ICR) mice	Charles River Laboratories Orient: Cesarean-derived (Institute of Cancer Research) mice
DEPT	Distortionless enhancement by polarisation transfer
DFL	Difucosyllactose
DNA	Deoxyribonucleic acid
DM	Dry matter
EU	Endotoxin unit
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed

FOF	Follow-on formula
FoodEx2	EFSA standardised food classification and description system
FS	Food supplements
FSMP	Food for special medical purposes
FSSC 22000	Food Safety System Certification 22000
GC/FID	Gas chromatography–flame ionisation detection
GGT	Gamma glutamyl transpeptidase
GLP	Good Laboratory Practice
GMO	EFSA Panel on Genetically Modified Organisms
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis Critical Control Points
HCT	Haematocrit
HGB	Haemoglobin
HiMO	Human-identical milk oligosaccharide
HMBC	Heteronuclear multiple-bond correlation
HMO	Human milk oligosaccharide
HPAEC-PAD	High-performance anion-exchange chromatography–pulsed amperometric detection
HPLC/FLD	High-performance liquid chromatography–fluorescence detection
HSQC	Heteronuclear single quantum correlation
ICP-MS	Inductively coupled plasma–mass spectrometry
IDF/RM	International Dairy Federation/Reviewed method
IF	Infant formula
ISO	International Organization for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
KCTC	Korean Collection of Type Cultures
Kgf	Kilogram-force
KMFDS	Korean Ministry of Food and Drug Safety
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
LNT	Lacto-*N*-tetraose
LNnT	Lacto-*N*-neotetraose
LOQ	Limit of quantification
Mod.	Modification of sample preparation methods
MS	Mass spectrometry
NANA	*N*-acetyl-D-neuraminic acid
ND	Not detected
NDA	EFSA Panel on Nutrition, Novel Foods and Food Allergens
NF	Novel food
NMR	Nuclear magnetic resonance spectroscopy
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
Ph. Eur.	European Pharmacopeia
qPCR	Quantitative polymerase chain reaction
QPS	Qualified presumption of safety
RBC	Red blood cells
RH	Relative humidity
ROESY	Rotating-frame nuclear Overhauser effect spectroscopy
SD	Standard deviation
TG	Test guidelines
TOCSY	Total correlation spectroscopy
TS	Technical specification
US FDA	US Food and Drug Administration
US FDA BAM	US Food and Drug Administration – Bacteriological Analytical Manual
USP	United States Pharmacopeia
WGS	Whole genome sequence
W/W	Weight per weight

Appendix A – Toxicity report (90-day repeated dose oral toxicity study; Unpublished Study, Biotoxtech Study No. B18673)

Parameter	Exposure (days)	Sex	Dose Group (mg/kg body weight)			
			Control	2,500 mean ± SD	5,000 mean ± SD	7,500 mean ± SD
Body weight (g)	4	M	27.6 ± 1.7	28.6 ± 2.4	25.9 ± 1.5	26.0 ± 0.8^ (−5.79%)
		F	26.2 ± 1.4	27.3 ± 2.9	25.9 ± 1.3	25.2 ± 1.7
	11	M	47.5 ± 2.8	49.8 ± 3.5	44.7 ± 2.6* (−5.89%)	45.2 ± 1.8
		F	44.9 ± 2.9	47.3 ± 5.7	45.2 ± 3	44.5 ± 2.4
Food consumption (g/day)	16	M	11.8 ± 3.3	11.1 ± 2.8	10.2 ± 1.1	10.0 ± 1.3
		F	11.0 ± 1.4	11.1 ± 1.6	10.3 ± 1.6	9.5 ± 0.9* (−13.64%)
Hindlimb grip strength (kgf)	90	M	0.584 ± 0.061	0.603 ± 0.065	0.552 ± 0.055	0.569 ± 0.075
		F	0.484 ± 0.068	0.408 ± 0.060* (−15.70%)	0.412 ± 0.058* (−14.88%)	0.405 ± 0.062* (−16.32%)
Clinical signs						
Soft stool (incidence)	–	M	0/15	0/10	0/10	15/15
	–	F	0/15	0/10	0/10	15/15
Diarrhoea (incidence)	–	M	0/15	0/10	0/10	14/15
	–	F	0/15	0/10	0/10	13/15
Haematological parameters						
Monocytes (%)	90	M	7.6 ± 1.6	9.7 ± 2.8	8.4 ± 2.4	10.6 ± 2.3* (+39.47%)
		F	7.4 ± 2.3	6.9 ± 2.0	6.7 ± 2.4	6.5 ± 0.9
Eosinophils (%)	90	M	1.2 ± 0.4	1.1 ± 0.3	0.9 ± 0.2	1.2 ± 0.5
		F	1.3 ± 0.4	1.2 ± 0.4	0.9 ± 0.2* (−30.77%)	1.1 ± 0.4
Prothrombin time (sec)	90	M	18.5 ± 0.8	17.5 ± 1.1	17.6 ± 0.9	17.9 ± 0.9
		F	18.5 ± 0.7	18 ± 0.8	17.7 ± 0.7* (−4.32%)	17.5 ± 0.7* (−5.41%)
RBC (x10^6/μL)	118(a)	M	8.68 ± 0.47	–	–	8.08 ± 0.53
		F	8.22 ± 0.22	–	–	7.71 ± 0.14 (−6.20%)
HGB (g/dL)	118(a)	M	15.5 ± 0.4	–	–	14.8 ± 0.7
		F	15.8 ± 0.2	–	–	14.8 ± 0.3* (−6.33%)
HCT (%)	118(a)	M	43.7 ± 0.6	–	–	41.9 ± 1.4
		F	43.9 ± 0.7	–	–	41.5 ± 0.6* (−5.47%)
Reticulocytes (%)	118(a)	F	2.43 ± 0.14	–	–	2.81 ± 0.21* (+15.64%)
Clinical chemistry parameters						
Creatinine (mg/dL)	90	M	0.45 ± 0.06	0.45 ± 0.04	0.41 ± 0.03	0.40 ± 0.04* (−11.11%)
		F	0.47 ± 0.03	0.51 ± 0.04	0.46 ± 0.04	0.47 ± 0.02
Triglycerides (mg/dL)	90	M	32 ± 15	69 ± 35^^ (+115.63%)	58 ± 59	66 ± 56
		F	17 ± 7	18 ± 10	20 ± 7	40 ± 43

Parameter	Exposure (days)	Sex	Dose Group (mg/kg body weight) Control	2,500 mean ± SD	5,000 mean ± SD	7,500 mean ± SD
Serum phosphorus (mg/dL)	90	M	6.25 ± 0.26	6.61 ± 0.50	6.79 ± 0.38* (+8.64%)	6.82 ± 0.63* (+9.12%)
		F	5.18 ± 0.67	5.57 ± 0.41	5.33 ± 0.40	4.95 ± 0.45
Serum chloride (mmol/L)	90	M	106.2 ± 0.8	105.8 ± 1.0	105.8 ± 1.9	104.5 ± 1.1* (−1.60%)
		F	108.8 ± 1.1	108.2 ± 0.9	107.8 ± 1.7	107.2 ± 1.5
ALT (U/L)	90	M	24.6 ± 6.7	23.5 ± 4.9	22.9 ± 5.3	32.4 ± 25.6
		F	23.1 ± 6.0	18.4 ± 4.7	16.4 ± 2.6* (−29.00%)	19.7 ± 6.0
GGT (U/L)	90	M	0.27 ± 0.17	0.18 ± 0.11	0.24 ± 0.13	0.22 ± 0.08
		F	0.53 ± 0.24	0.51 ± 0.20	0.42 ± 0.30	0.24 ± 0.11* (−54.72%)
Total cholesterol (mg/dL)	90	M	90 ± 13	97 ± 26	75 ± 14	74 ± 21
		F	70 ± 14	88 ± 16* (+25.71%)	94 ± 21** (+34.29%)	98 ± 14** (+40.00%)
A/G ratio	118 (a)	M	0.71 ± 0.05	–	–	0.66 ± 0.07
		F	0.76 ± 0.04	–	–	0.84 ± 0.06* (+10.53%)
Urinalysis						
Urine volume (mL)	90	M	11.6 ± 2.7	14.8 ± 6.2	13.6 ± 5.3	14 ± 4.3
		F	9.3 ± 2.4	5.5 ± 1.3* (−40.86%)	5.9 ± 1.8 (−36.56%)	5.2 ± 2.8* (−44.09%)
Organ weight						
Absolute brain weight (g)	118(a)	M	2.19 ± 0.16	–	–	2.17 ± 0.08
		F	2.02 ± 0.06	–	–	1.89 ± 0.07* (−6.44%)
Relative liver weight (g/100 g body weight)	90	M	2.74 ± 0.27	3.10 ± 0.24** (+13.14%)	2.91 ± 0.24	3.05 ± 0.27* (+11.31%)
	90	F	2.52 ± 0.19	2.87 ± 0.95	2.75 ± 0.18* (+9.13%)	2.91 ± 0.27* (+15.48%)
Relative thymus weight (g/100 g body weight)	118(a)	M	0.06 ± 0.01	–	–	0.05 ± 0.01* (−16.67%)
		F	0.09 ± 0.02	–	–	0.09 ± 0.02

M: male; F: female; Steel-test ^: $p < 0.05$; ^^: $p < 0.01$; Dunnett's t-test *: $p < 0.05$; **: $p < 0.01$.
RBC: red blood cells; HGB: haemoglobin; HCT: haematocrit; ALT: alanine aminotransferase; GGT: gamma glutamyl transpeptidase.
(a): End of recovery period (n = 5, limited to control and high dose groups).