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Safety of 3-fucosyllactose (3-FL) produced by a derivative strain of *Escherichia coli* K-12 DH1 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 3-fucosyllactose (3-FL) as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The NF is mainly composed of the human-identical milk oligosaccharide (HiMO) 3-FL, but it also contains D-lactose, L-fucose, 3-fucosyllactulose and a small fraction of other related saccharides. The NF is produced by fermentation by a genetically modified strain (*Escherichia coli* K-12 DH1 MDO MAP1834) of *E. coli* K-12 DH1 (DSM 4235). The information provided on the manufacturing process, composition and specifications of the NF does not raise safety concerns. The applicant intends to add the NF to a variety of foods, including infant formula and follow-on formula, food for special medical purposes and food supplements (FS). The target population is the general population. The anticipated daily intake of 3-FL from both proposed and combined (authorised and proposed) uses at their respective maximum use levels in all population categories does not exceed the highest intake level of 3-FL from human milk in infants on a body weight basis. The intake of 3-FL in breastfed infants on a body weight basis is expected to be safe also for other population groups. The intake of other carbohydrate-type compounds structurally related to 3-FL is also considered of no safety concern. FS are not intended to be used if other foods with added 3-FL or human milk are consumed on the same day. The Panel concludes that the NF is safe under the proposed conditions of use.

Keywords: 3-fucosyllactose, 3-FL, human milk oligosaccharide, HMO, HiMO, novel food, safety

Requestor: European Commission

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1. Introduction

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

On 25 March 2021, the company Glycom A/S submitted a request to the Commission in accordance with Article 10 of Regulation (EU) 2015/2283[1] to place on the EU market 3-fucosyllactose (3-FL) as a novel food (NF).

3-FL is intended to be used in a number of foods and in food supplements (FS) as defined in Directive 2002/46/EC.

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 3-FL as a NF.

In addition, EFSA is requested to include in its scientific opinion a statement in line with the requirements of Article 26(2)(c) of Regulation (EU) 2015/2283, as to whether and if so to what extent, the proprietary data for which the applicant is requesting data protection was used in elaborating the opinion.

1.2. Additional information

3-FL is included in the Union list of authorised NFs (Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017[2]) when produced by genetically modified strains of *Escherichia coli* K-12 MG1655 or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2021, 2022a). 2′-Fucosyllactose (2′-FL), a constitutional isomer of 3-FL, is also included in the Union list of authorised NFs when produced by chemical synthesis (EFSA NDA Panel, 2015a) or by genetically modified strains of *E. coli* K-12 DH1 or *E. coli* BL21 (DE3). A 2′-FL/difucosyllactose (DFL) mixture produced by a genetically modified strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019a) is also included in the Union list of authorised NFs. Moreover, the extensions of use in FS for infants of 2′-FL and 2′-FL/DFL mixture, both produced by genetically modified strains of *E. coli* K-12 DH1, and the safety of 2′-FL produced by a genetically modified strain (APC199) of *Corynebacterium glutamicum* ATCC 13032, have recently been assessed by EFSA with positive outcomes (EFSA NDA Panel, 2022b,c,d).

Since 2015, several scientific opinions with positive outcomes 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) and 2′-FL produced by a genetically modified strain (APC199) of *C. glutamicum* ATCC 13032 (EFSA NDA Panel, 2022d);
- Chemically synthesised lacto-*N*-neotetraose (LNnT) (EFSA NDA Panel, 2015b) and LNnT produced by genetically modified 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 by genetically modified strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2022b);
- Chemically synthesised *N*-acetyl-D-neuraminic acid (NANA) (EFSA NDA Panel, 2017);
- 2′-FL/DFL mixture produced by a genetically modified strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019a);
- Lacto-*N*-tetraose (LNT) produced by genetically modified strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019b) or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022e);
- Extension of use in FS for infants of 2′-FL/DFL mixture and LNT produced by genetically modified strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2022c);
- 3-FL produced by genetically modified strains of *E. coli* K-12 MG1655 (EFSA NDA Panel, 2021) or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022a);

[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) N0 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 2013/0435 (COD). OJ L 327, 11.12.2015, pp. 1–22.

[2] 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 351/72, 30.12.2017.

- 3′-Sialyllactose (3′-SL) sodium salts produced by genetically modified strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020b) or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022f;
- 6′-Sialyllactose (6′-SL) sodium salts produced by genetically modified strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020c) or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022g).

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 an EFSA request 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[3].

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) production process; (iii) information on the genetically modified production strain; (iv) composition and stability of the NF; (v) literature search; (vi) intake assessment; and (vii) toxicological information.

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, food for special medical purposes (FSMP) and total diet replacement for weight control are laid down in Regulation (EU) No 609/2013[4] and, respectively, in Commission Delegated Regulation (EU) 2017/1798[5] (total diet replacement for weight control), in Commission Delegated Regulation (EU) 2016/128[6] (FSMP) and in Commission Delegated Regulation (EU) 2016/127[7] (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] 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.

[4] 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.

[5] 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.

[6] 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.

[7] 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.

3. Assessment

3.1. Introduction

The NF, which is the subject of the application, contains 3-FL as primary constituent (≥ 90.0% w/w dry matter (DM)), a fucosylated neutral oligosaccharide consisting of L-fucose linked via an α-(1–3) bond to the D-glucose unit of D-lactose. 3-FL has been identified as a relevant component of the complex fraction of oligosaccharides naturally occurring in mammalian milk, with the highest concentration present in human milk, and therefore is typically denominated as a human milk oligosaccharide (HMO). The Panel notes that although 3-FL is the major component of the NF, it also contains D-lactose, L-fucose, 3-fucosyllactulose and a small fraction of other related saccharides. The NF is produced by fermentation by a genetically modified strain (*E. coli* K-12 DH1 MDO MAP1834) of *E. coli* K-12 DH1 (DSM 4235).

The NF is proposed to be used in IF, FOF, FSMP and total diet replacements for weight control, as defined in Regulation (EU) No 609/2013, FS as defined in Directive 2002/46/EC, beverages and in a variety of other foods (e.g., dairy products, cereals). The target population is the general population.

3-FL produced by genetically modified strains of *E. coli* K-12 MG1655 or *E. coli* BL21 (DE3) has been previously assessed by EFSA as a NF with positive outcomes (EFSA NDA Panel, 2021, 2022a). In addition, other HiMOs produced by genetically modified strains of the same parental strain *E. coli* K-12 DH1, i.e., 2′-FL, LNnT, 2′-FL/DFL mixture (EFSA NDA Panel, 2019a), LNT (EFSA NDA Panel, 2019b), 3′-SL sodium salt (EFSA NDA Panel, 2020b) and 6′-SL sodium salt (EFSA NDA Panel, 2020c), have been authorised as NFs in the European Union (Commission Implementing Regulation (EU) 2017/2470).

According to Article 3(2)(a) of Regulation (EU) 2015/2283, the 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 3-FL (≥ 90.0% w/w DM), which is one of the most abundant neutral fucosylated HMOs (Erney et al., 2001; Thurl et al., 2017). The NF also contains L-fucose (≤ 1.0% w/w), D-lactose (≤ 5.0% w/w), 3-fucosyllactulose (≤ 1.5% w/w) and a small fraction of other related saccharides (sum of other carbohydrates ≤ 5.0% w/w). It is produced by fermentation by a genetically modified strain (*E. coli* K-12 DH1 MDO MAP1834) of *E. coli* K-12 DH1 (DSM 4235). 3-FL is a trisaccharide consisting of L-fucose linked via an α-(1–3) bond to the D-glucose moiety of D-lactose (Table 1 and Figure 1). 3-FL is a constitutional isomer of 2′-FL, which contains the same monosaccharide moieties as those present in 3-FL but with the linkage between L-fucose and the D-galactose moiety of D-lactose being α-(1–2′) instead of α-(1–3).

Table 1: Chemical identity of 3-FL

Chemical substance	
Chemical (IUPAC) name	(2*S*,3*S*,4*R*,5*S*,6*S*)-2-[(3*R*,4*R*,5*R*,6*R*)-2,3-dihydroxy-6-(hydroxymethyl)-5-[(2*S*,3*R*,4*S*,5*R*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyoxan-4-yl]oxy-6-methyloxane-3,4,5-triol
Common name	3-Fucosyllactose
Abbreviations	3-FL, 3FL, 3F, 3fl, 3-fl
Alternative chemical names	• β-D-Gal-(1–4)-[α-L-Fuc-(1–3)]-D-Glc
	• α-L-Fuc-(1→3)-[β-D-Gal-(1→4)]-D-Glc
	• β-D-Galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-D-glucopyranose
	• α-L-Fucosypyranosyl-(1→3)-[β-D-galactopyranosyl-(1→4)]-D-glucopyranoside
CAS number	41312-47-4
Molecular formula	$C_{18}H_{32}O_{15}$
Molecular mass	488.44 Da

CAS: Chemical Abstracts Service; IUPAC: International Union of Pure and Applied Chemistry.

Several analyses were performed on the NF in order to confirm the structure of 3-FL, the major constituent of the NF.

The structure of (non-crystalline) 3-FL was determined by mono-dimensional (1D) nuclear magnetic resonance (NMR) spectroscopy, including ^{1}H, ^{13}C and ^{13}C-DEPT-Q (distortionless enhancement by polarisation transfer with retention of quaternaries) spectra and two-dimensional (2D) NMR spectroscopy, including g-DQFCOSY (gradient double-quantum-filtered correlation spectroscopy), g-HSQC (gradient heteronuclear single quantum coherence) and g-HMBC (gradient heteronuclear multiple bond coherence) spectra, by comparison to a commercially available authentic specimen[8]. The relevant coupling constants measured by ^{1}H NMR together with the correlations evidenced on the 2D NMR spectra confirmed: (i) the α configuration of L-fucose and the β configuration of the D-galactose moiety of D-lactose; (ii) the α-(1″-3) bond between L-fucose (Fuc-H-1″) and the D-glucose (Glc-C-3) moiety of D-lactose and (iii) the β-(1′-4) link between the D-galactose (Gal-H-1′) and D-glucose (Glc-C-4) moieties of D-lactose. These results are consistent with the full assignment of the NMR spectra of 3-FL reported in the literature (Ishizuka et al., 1999).

The molecular structure of 3-FL (non-crystalline and crystalline) was corroborated by high-performance liquid chromatography – electrospray ionisation – tandem mass spectrometry (HPLC–ESI–MS/MS) based on its collision-induced dissociation (CID) fragmentation pattern, by comparison to a commercially available high-purity analytical standard, which allowed to differentiate between 3-FL α-(1″-3) and 2′-FL α-(1″-2′). The mass fragmentation pattern is consistent with that reported in the literature (Zaia, 2004; Kailemia et al., 2014; Wang et al., 2016; Yamagaki and Makino, 2017; Mank et al., 2019).

The identity of 3-FL (non-crystalline and crystalline) was also corroborated by high-performance anion-exchange chromatography – pulsed amperometric detection (HPAEC–PAD) by comparison to a commercially available high-purity analytical standard.

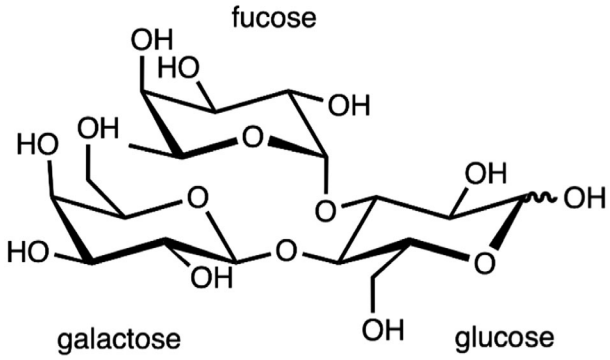

Figure 1: Chemical structure of 3-FL (EFSA NDA Panel, 2021)

On the basis of the spectroscopic and chromatographic evidence, the Panel considers that the 3-FL present in the NF produced by *E. coli* K-12 DH1 MDO MAP1834 is identical to the 3-FL in human milk and therefore, it is regarded as being a HiMO.

3.3. Production process

According to the information provided, the NF is produced in line with Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Points (HACCP) principles. The production process (including all used processing aids, raw materials, unit operations and filter aids), as well as food safety management system comply with the following standards and certifications: Food Safety Systems Certification (FSSC) 22000 and International Organization for Standardization (ISO) 9001.

The NF is produced by fermentation by the genetically modified strain *E. coli* K-12 DH1 MDO MAP1834. D-Lactose and D-glucose (alternatively, D-sucrose or glycerol) are converted into 3-FL by the adapted cellular metabolism of the production strain, which uses D-glucose as an energy and carbon source and D-lactose as a substrate for 3-FL biosynthesis. The production microorganism is removed from the fermentation medium by ultrafiltration/diafiltration and microfiltration at the end of the fermentation process. A series of isolation, purification and concentration steps are then used to obtain

[8] 3′-FL isolated from human milk.

high-purity 3-FL in powder form. An optional crystallisation step with acetic acid may be included during downstream processing to generate a higher-grade product with reduced concentration of certain carbohydrate-type impurities.

The production strain *E. coli* K-12 DH1 MDO MAP1834 is a genetically modified derivative of the parental strain *E. coli* K-12 DH1 (*F- λ- gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44*), which was obtained by the applicant from the German Collection of Microorganisms and Cell cultures (DSMZ) (commercially available under DSM 4235). The parental strain *E. coli* K-12 DH1 is derived from *E. coli* K-12 by forced random mutagenesis. The whole genomes of *E. coli* K-12 and other closely derivative strains, including *E. coli* K-12 DH1, were sequenced and compared to other *E. coli* strains including pathogenic strains, which evidenced genomic differences in *E. coli* K-12 and its derivatives as compared to the pathogenic strains (Blattner et al., 1997; Lukjancenko et al., 2010). Although the species *E. coli* is considered not suitable for qualified presumption of safety (QPS) status (EFSA BIOHAZ Panel, 2023), the strain *E. coli* K-12 is considered as a safe and non-pathogenic or toxigenic microorganism widely used for biotechnological applications (Gorbach, 1978; OECD, 1986; Muhldorfer and Hacker, 1994; US EPA, 1997; ZKBS, 2021).

The production strain has been deposited at the DSMZ culture collection. A detailed description of the genetic modification steps applied to the parental strain *E. coli* K-12 DH1 (DSM 4235) to obtain the platform strain *E. coli* K-12 DH1 MDO (membrane-derived oligosaccharides) and the production strain *E. coli* K-12 DH1 MDO MAP1834 has been provided by the applicant. No residual DNA from the production strain was detected in the NF using three quantitative polymerase chain reaction (qPCR) assays targeting short sub-sequences of specific inserted genes, as well as a short sub-sequence of the 23S rRNA subunit of *E. coli*. The absence of both DNA and viable cells from the production strain in the NF 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

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

Batch-to-batch analyses showed that the NF consists of 3-FL as the main component (95.1% w/w DM[9]; 94.4% w/w DM[10] in non-crystalline batches and 95.7% w/w DM[11] in crystalline batches). The remaining constituents[12] include L-fucose (0.14% w/w[9]; 0.23% w/w[10]/0.05% w/w[11]), D-lactose (0.82% w/w[9]; 1.55% w/w[10]/0.10% w/w[11]) and 3-fucosyllactulose (0.25% w/w[9]; 0.43% w/w[10]/0.07% w/w[11]). Apart from the above-specified saccharides (96.2% w/w DM[9]; 96.7% w/w DM[10]/95.9% w/w DM[11]), the NF contains other carbohydrates individually present at low concentration (sum of other quantified carbohydrates, 1.22% w/w[9]; 0.94% w/w[10]/1.49% w/w[11]).

With regard to physico-chemical properties, the NF can be described as a white to off-white powder. The solubility in water was measured in two batches of the NF, one non-crystalline and one crystalline, according to the EFSA Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles (EFSA Scientific Committee, 2021), resulting in an average value of 732 g/L and 700 g/L, respectively.

[9] Average content in eight batches of the NF (four non-crystalline and four crystalline).
[10] Average content in four non-crystalline batches of the NF.
[11] Average content in four crystalline batches of the NF.
[12] 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.

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

Parameters	Non-crystalline batches				Crystalline batches				Method of analysis
	#1	#2	#3	#4	#5	#6	#7	#8	
Composition									
Specified saccharides[1] (% w/w DM)	96.3	98.5	96.4	95.4	93.8	95.9	97.4	96.5	HPAEC-PAD (validated internal method)
3-FL (% w/w DM)	93.4	97.5	92.1	94.6	93.2	95.8	97.4	96.5	
L-Fucose (% w/w)	0.13	0.16	0.39	0.23	0.11	0.03	< 0.03	< 0.03	
D-Lactose (% w/w)	2.15	0.29	3.40	0.36	0.30	< 0.03	< 0.03	< 0.03	
3-Fucosyllactulose (% w/w)	0.57	0.56	0.39	0.18	0.12	0.11	< 0.03	< 0.03	
Sum of other carbohydrates (% w/w)	0.46	0.73	0.82	1.76	2.70	1.52	0.75	0.98	HPAEC-PAD; HPLC-CAD (validated internal methods)
pH (5% solution, 20°C)	4.6	5.7	5.7	5.5	3.7	3.8	3.9	3.9	Ph. Eur. 9.2 2.2.3 (potentiometry)
Water (% w/w)	3.60	3.23	3.49	2.41	0.14	0.18	0.04	0.01	Karl Fischer titration (coulometric titration)
Ash, sulfated (% w/w)	< 0.01	< 0.01	< 0.01	0.10	< 0.01	< 0.01	< 0.01	< 0.01	Ph. Eur. 9.2 2.4.1.4 (gravimetry) Ph Eur. 6.7 04/2010:20414 (gravimetry)
Protein (% w/w)	< 0.0017	< 0.0017	< 0.0017	< 0.0017	< 0.0017	0.00405	< 0.0017	< 0.0017	Bradford assay (spectrophotometry)
Acetic acid[2] (% w/w)	–	–	–	–	0.5	0.3	0.1	0.1	Acetic acid assay kit (enzymatic, spectrophotometry)
Contaminants									
Arsenic (total) (mg/kg)	< 0.1	< 0.1	< 0.1	0.1	< 0.1	< 0.1	< 0.1	< 0.1	MSZ EN 13805:2015, EPA 6020A:2007 (ICP-MS) EN 13805 m:2014 EN ISO 17294 m:2016 (ICP-MS)
Cadmium (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Lead (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Mercury (mg/kg)	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	
Aflatoxin M1 (μg/kg)	< 0.020	< 0.020	< 0.020	< 0.020	–	–	< 0.020	< 0.020	LC–MS/MS (internal method)
Microbial parameters									
Total plate count (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	MSZ EN ISO 4833-1:2014 (colony count)
Yeasts (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	MSZ ISO 21527-2:2013 (colony count)
Moulds (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	MSZ ISO 21527-2:2013 (colony count)
Enterobacteriaceae (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	MSZ EN ISO 21528-2:2017 or NMKL 144 (colony count)

Parameters	Non-crystalline batches				Crystalline batches				Method of analysis
	#1	#2	#3	#4	#5	#6	#7	#8	
Enterobacteriaceae (in 10 g)	ND	ND	ND	ND	ND	ND	ND	ND	MSZ EN ISO 21528-1:2017 (detection or qualitative method)
Salmonella spp. (in 25 g)	ND	ND	ND	ND	ND	ND	ND	ND	MSZ EN ISO 6579-1:2017 (detection or qualitative method) AFNOR BRD 7/11–12/5 (detection or qualitative method)
Cronobacter spp. (in 10 g)	ND	ND	ND	ND	ND	ND	ND	ND	MSZ EN ISO 22964:2017 (detection or qualitative method)
Listeria monocytogenes (in 25 g)	ND	ND	ND	ND	ND	ND	ND	ND	MSZ EN ISO 11290-1:2017 (detection or qualitative method)
Presumptive *Bacillus cereus* (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	MSZ EN ISO 7932:2005 (colony count)
Endotoxins (EU/mg)	0.0066	0.0122	0.0016	< 0.00025	0.0498	0.0110	0.0007	0.0077	Ph. Eur. 2.6.14 (gel-clot technique)

'–': Not reported; 3-FL: 3-Fucosyllactose; AFNOR: Association Francaise de Normalisation; BRD: Bacteriology Reference Department; CFU: Colony forming units; DM: Dry matter; EN: European norm; EPA: Environmental Protection Agency; EU: Endotoxin units; HPAEC–PAD: High-performance liquid chromatography – electrospray ionisation – tandem mass spectrometry; HPLC-CAD: High-performance liquid chromatography – charged aerosol detection; ICP-MS: Inductively coupled plasma – mass spectrometry; ISO: International Organisation for Standardisation; LC–MS/MS: Liquid chromatography – tandem mass spectrometry; m: Modification of analytical methods; MSZ: Polish Ministry of Foreign Affairs; ND: Not detected; NMKL: Nordisk Metodikkomite for Levnedsmidler; Ph. Eur.: European Pharmacopoeia; w/w: Weight per weight.

1 Specified saccharides include 3-FL, D-lactose, L-fucose and 3-fucosyllactulose.

2 Only relevant for batches of the NF crystallised with acetic acid.

The Panel considers that the information provided on the composition is sufficient for characterising the NF and does not raise safety concerns.

3.4.1. Stability

The applicant carried out a 5-year real-time stability study (25°C, 60% relative humidity (RH)) (ongoing) and a 2-year stability study under accelerated conditions (40°C, 75% RH), with two batches of the NF (one non-crystalline and one crystalline). Results up to 24 months were provided for both stability studies and batches of the NF, including sensory parameters and carbohydrate and water content. Microbial parameters were also monitored on both batches of the NF up to 12 and 24 months under normal and accelerated storage conditions, respectively.

No appreciable changes in the organoleptic properties, carbohydrate, 3-FL and moisture content were observed up to 24 months of storage under normal and accelerated conditions for both batches of the NF. Microbial parameters on both batches of the NF were also below the respective limits of detection over the 12-month and 24-month storage period under normal and accelerated conditions, respectively.

The applicant also provided the results of different stressed/forced stability studies with a crystalline batch of the NF in solid state or aqueous solution, as follows:

- No degradation products were detected when the NF in powdered solid state was stored at 80°C for 28 days at two different levels of air humidity.
- Four potential pH-dependent degradation pathways were proposed as a result of the tests carried out in aqueous solution (10 mg/mL) at 60°C and different pH conditions (unbuffered; buffered at 3.0, 5.0, 6.8, and 9.0; in 0.1 N HCl; in 0.01 N NaOH) up to 28 days (or until significant degradation):
    - at pH < 5 (i) hydrolysis to lactose and fucose and/or (ii) isomerisation to 6′-β-fucosyllactose;
    - at pH > 5 (iii) isomerisation to 3-fucosyllactulose and/or (iv) 'peeling reaction' characteristic to carbohydrates substituted in position 3 and related to the terminal anomeric carbon, with fucose and galactose being the primary products detected in about equal amounts, and the glucose moiety being degraded to several products.
- No degradation products were identified from the studies conducted in aqueous solution (10 mg/mL) at room temperature for 24 h in presence of oxidising agents, i.e., 0.1% hydrogen peroxide or 4,4′-azobis-(4-cyanovaleric acid) (ACVA) in 1:0.1 M ratio.

The applicant also referred to stability studies on 2'-FL (EFSA NDA Panel, 2015a, 2019a) and proposed a 5-year shelf-life under ambient conditions for the NF.

The Panel considers that the available data provided sufficient information with respect to the stability of the NF for 24 months.

Stability of the NF under the intended conditions of use

A 3-year stability study (ongoing) was conducted in a commercially representative powdered IF supplemented with the NF (1% w/w DM 3-FL) and stored at 5°C, 25°C/60% RH, 30°C/65% RH or 40°C/75% RH. Interim results, including the 3-FL content and microbial parameters, showed that 3-FL is stable up to 12 months under the above-mentioned storage conditions.

Additional stability studies demonstrated that 3-FL is stable in formulations representative of commercial food products on the market under typical processing and storage conditions for such products, as follows: cereal bars including or not a heating step over 100°C (up to 3 months at ambient conditions); pasteurised juice drink (up to 28 days at 5°C), pasteurised ready-to-drink milkshake (up to 14 days at 5°C), UHT ready-to-drink milkshake (up to 28 days at 5°C) and fruit yoghurt (up to 21 days at 5°C).

The applicant also referred to the study by Christensen et al. (2020), where the stability of high-purity 3-FL and 2'-FL standards was demonstrated in pasteurised whole milk and yoghurt at 5°C for 22 and 36 days, respectively, and in UHT milk at room temperature for 3 months. In addition, the stability of the authorised 3-FL and 2'-FL has been demonstrated in various food matrices, including IF, whole/UHT milk, yoghurt, ready-to-drink flavoured milk, citrus fruit beverages and cereal bars (EFSA NDA Panel, 2015a, 2019a, 2021, 2022a).

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: 3-FL is a white to off-white powder produced by microbial fermentation and further isolated, purified and concentrated.	
Source: A genetically modified strain (*Escherichia coli* K-12 DH1 MDO MAP1834) of *E. coli* K-12 DH1.	
Parameter	**Specification**
Composition	
Specified saccharides[1] (% w/w DM)	≥ 92.0
3-FL (% w/w DM)	≥ 90.0
L-Fucose (% w/w)	≤ 1.0
D-Lactose (% w/w)	≤ 5.0
3-Fucosyllactulose (% w/w)	≤ 1.5
Sum of other carbohydrates (% w/w)	≤ 5.0
pH (5% solution, 20°C)	3.2–7.0
Water (% w/w)	≤ 6.0
Ash (% w/w)	≤ 0.5
Acetic acid[2] (% w/w)	≤ 1.0
Protein (% w/w)	≤ 0.01
Contaminants	
Arsenic	≤ 0.2 mg/kg
Aflatoxin M1	≤ 0.025 μg/kg
Microbial parameters	
Total plate count (CFU/g)	≤ 1,000 CFU/g
Yeasts and moulds (CFU/g)	≤ 100
Enterobacteriaceae	ND in 10 g
Salmonella	ND in 25 g
Cronobacter spp.	ND in 10 g
Listeria monocytogenes	ND in 25 g
Presumptive *Bacillus cereus* (CFU/g)	≤ 50
Endotoxins (EU/mg)	≤ 10

3-FL: 3-Fucosyllactose; CFU: Colony forming units; DM: Dry matter; EU: Endotoxin units; ND: Not detected; w/w: Weight per weight.
1 Specified saccharides include 3-FL, D-lactose, L-fucose and 3-fucosyllactulose.
2 Only relevant for the NF crystallised with acetic acid.

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, 3-FL produced with genetically modified strains of *E. coli* K-12 MG1655 or *E. coli* BL21(DE3) has been previously assessed by EFSA as a NF (EFSA NDA

Panel, 2021, 2022a,b,c,d,e,f,g) and authorised in the European Union (Commission Implementing Regulation (EU) 2021/2029[13] and 2023/52[14]).

The history of human exposure to 3-FL refers to breastfed infants who are naturally exposed to HMO. 3-FL was found in the human milk of most women (> 96%) in 10 countries studied (Erney et al., 2000, 2001). The Panel also notes that 3-FL is among the most abundant HMOs and shows increasing levels over the course of lactation (Thurl et al., 2017; Samuel et al., 2019; Soyyılmaz et al., 2021). Oligosaccharides in bovine milk are more than 20 times less concentrated than in human milk and the vast majority (~ 90%) is composed of acidic oligosaccharides (Bode, 2012; Aldredge et al., 2013; Albrecht et al., 2014). Wang et al. (2020) have reported mean concentrations of 3-FL of 0.09 and 0.07 g/L in bovine milk and goat milk, respectively.

3.6.2. Intake of 3-FL from human milk

As reported in previous EFSA opinions (EFSA NDA Panel, 2019b, 2020a,b, 2021, 2022c), human milk contains a family of structurally related oligosaccharides, known as HMOs, which is the third largest fraction of solid components. The highest concentrations of HMOs occur in human colostrum (20–25 g/L), and concentrations between 5 and 20 g/L occur in mature human milk (Thurl et al., 2010; Bode, 2012; Gidrewicz and Fenton, 2014; Urashima et al., 2018). HMOs' concentrations and composition vary across mothers and over the course of lactation. 3-FL belongs to the subfraction of neutral fucosylated HMOs, characterised by the presence of L-fucose. This fraction accounts for up to 80% of the total HMO concentration (Thurl et al., 2010; Rijnierse et al., 2011; Bode, 2012).

Several publications on HMOs and 3-FL show a wide variability of concentrations in human milk. From the systematic review by Thurl et al. (2017), the reported range of mean concentrations of 3-FL in human milk is 0.24–1.24 g/L for 0–100 days lactation, with maximum means of 0.34–1.44 g/L and increasing concentrations over time. In another study (Erney et al., 2001), an average concentration of 1.39 g/L and a maximum concentration of 3.92 g/L for 3-FL have been reported.

Other publications reported maximum concentrations in European human milks up to 3.4 g 3-FL/L (average 0.2–2.3 g/L; Austin et al., 2019) or up to 5.7 g 3-FL/L (average 0.4–1.2 g/L; Samuel et al., 2019). In a recent review (Soyyılmaz et al., 2021), a mean of mean concentrations and a maximum mean of 0.92 and 2.57 g 3-FL/L, respectively, were recorded in mature milk (> 90 days).

In consideration of the large and recent data set used in this review (Soyyılmaz et al., 2021), and aligned with the recent 3-FL EFSA opinion (EFSA NDA Panel, 2022a), the Panel decided to use the values corresponding to the mean of means (0.92 g/L) and the maximum mean (2.57 g/L) as representative of the average natural concentrations found in human milk.

Based on these reported concentrations of 3-FL in human milk and considering the average and high daily intakes of human milk (800 mL and 1,200 mL, respectively) for infants from 0 to 6 months (EFSA NDA Panel, 2013), the daily intake levels of 3-FL from human milk for a 6.7 kg body weight (bw) infant (EFSA Scientific Committee, 2012) have been calculated (Table 4). This default body weight used by the NDA Panel is for an infant of 3–6 months of age, who is more likely than younger infants to consume these volumes of human milk.

Table 4: Estimated daily intake levels of 3-FL from average (800 mL) and high (1,200 mL) human milk intake for infants of 6.7 kg bw, based on mean of means and maximum mean concentration of 3-FL of 0.92 g/L and 2.57 g/L, in mature human milk (lactation day > 90) (Soyyılmaz et al., 2021) (EFSA NDA Panel, 2022a)

	Daily intake levels (mg/kg bw) from 800 mL of human milk		**Daily intake levels (mg/kg bw) from 1,200 mL of human milk**	
	Mean concentration	**High concentration**	**Mean concentration**	**High concentration**
3-FL	110	307	165	**460**

3-FL: 3-Fucosyllactose; bw: Body weight.

[13] Commission Implementing Regulation (EU) 2021/2029 of 19 November 2021 authorising the placing on the market of 3-fucosyllactose (3-FL) as a novel food under Regulation (EU) 2015/2283 of the European Parliament and of the Council and amending Commission Implementing Regulation (EU) 2017/2470; OJ L 415, 22.11.2021, pp. 9–14.

[14] Commission implementing regulation (EU) 2023/52 of 4 January 2023 authorising the placing on the market of 3-fucosyllactose produced by a derivative strain of Escherichia coli BL21(DE3) as a novel food and amending Implementing Regulation (EU) 2017/2470; OJ L3; pp. 1–7.

3.7. Proposed uses and use levels and anticipated intake

3.7.1. Target population

The target population proposed by the applicant is the general population.

3.7.2. Proposed uses and use levels

The NF is proposed to be used as an ingredient in various food categories, including IF and FOF. These food products, defined using the FoodEx2 hierarchy, and the proposed maximum use levels, are reported in Table 5.

The applicant also intends to market the NF for use in FS as defined in Directive 2002/46/EC. Specifically, maximum daily intakes of 2 g/day for infants and young children, or 4 g/day for the other population groups have been proposed.

For FSMP, the applicant did not propose maximum use levels and the Panel considers that the maximum use levels of the NF should not be higher than the maximum levels specified for the proposed food uses or the maximum daily intake proposed for FS.

FS are not intended to be used in infants and young children if other foods with added NF or human milk are consumed on the same day.

Table 5: Food categories according to FoodEx2 hierarchy and maximum use levels of the NF intended by the applicant

FoodEx2 code	FoodEx2 level	Food category	Proposed max. use levels (mg NF/100 g)
			Expressed as 3-FL
A02LV	5	Cow milk	200
A0CXA	5	European buffalo milk	200
A02MC	5	Sheep milk	200
A02MB	4	Goat milk	200
A02MV	3	Butter milk	200
A02NQ	4	Yoghurt drinks, including sweetened and/or flavoured variants	200
A02NR	4	Probiotic milk-like drinks	200
A02NV	5	Kefir	200
A02NE	4	Yoghurt	400
A00EY	3	Cereal bars	2,500
A03PZ	4	Infant formulae, powder	1,400[(a)]
A03QE	4	Infant formulae, liquid	175[(a)]
A03QK	4	Follow-on formulae, powder	1,400[(a)]
A0EQQ	4	Follow-on formulae, liquid	175[(a)]
A03RN	3	Fruit and vegetable juices and nectars specific for infants and young children	200
A03RP	3	Special food for children's growth	200
A03RT	4	Total daily diet replacement for weight reduction	2,500
A0EQN	4	Soft drinks with minor amounts of fruits or flavours	125

3-FL: 3-Fucosyllactose; NF: Novel food.

(a) Relevant dilution factors (EFSA, 2018) have been used to calculate intake estimates applying the FoodEx2 food classification and description system.

3.7.3. Anticipated intake of the NF

Anticipated intake of 3-FL from the proposed use level of the NF in IF in infants up to 16 weeks of age

IF is expected to be the only food consumed by infants aged 0–16 weeks who are not breastfed. A high consumption of IF has been estimated to be 260 mL/kg bw per day for infants aged 0–16 weeks

(EFSA Scientific Committee, 2017). Based on the maximum proposed use level of the NF (1.75 g 3-FL/L in IF), the high intake of the NF from IF alone is estimated to be 455 mg 3-FL/kg bw per day.

The Panel notes that the anticipated daily intake of the NF from the consumption of IF (only) does not exceed the estimated mean highest daily intake of 3-FL of 460 mg/kg bw per day in breastfed infants (Table 4).

Anticipated intake of 3-FL from the proposed uses and use levels of the NF

EFSA performed an intake assessment of the anticipated daily intake of the NF based on the proposed uses and maximum use levels by the applicant (Table 5), using individual data from the EFSA Comprehensive European Food Consumption Database (EFSA, 2011). The lowest and highest mean and 95th percentile anticipated daily intake of the NF (on a mg/kg body weight (bw) basis), among the EU dietary surveys, are presented in Table 6.

The estimated daily intake of the NF for each population group from each EU dietary survey is available in the excel file annexed to this scientific opinion under the Supporting Information section.

Table 6: Intake estimate resulting from the use of 3-FL as an ingredient in the intended food categories at the maximum proposed use levels

Population group	Age (years)	Mean intake (mg/kg bw per day)		P95th intake (mg/kg bw per day)	
		Lowest[(a)]	Highest[(a)]	Lowest[(b)]	Highest[(b)]
Infants	< 1	40	172	134	387
Young children[(c)]	1 to < 3	38	102	99	189
Other children	3 to < 10	17	65	41	113
Adolescents	10 to < 18	3	25	14	54
Adults[(d)]	≥ 18	10	13	23	32

3-FL: 3-Fucosyllactose; bw: Body weight.

(a) Intakes are assessed for all EU dietary surveys available in the food comprehensive database on 16 February 2023. The lowest and the highest averages observed among all EU surveys are reported in these columns.

(b) Intakes are assessed for all EU dietary surveys available in the food comprehensive database on 16 February 2023. The lowest and the highest P95th observed among all EU surveys are reported in these columns (P95th based on less than 60 individuals are not considered).

(c) Referred as 'toddlers' in the EFSA food consumption comprehensive database (EFSA, 2011).

(d) Includes elderly, very elderly, pregnant and lactating women.

The Panel notes that the content of 3-FL in the NF accounts for about 95.1%; therefore, the figures that are calculated considering a 100% purity slightly overestimate the actual intake. The Panel also notes that the anticipated daily intake of the NF from the proposed uses and maximum use levels does not exceed the estimated mean highest daily intake of 460 mg/kg bw of 3-FL in breastfed infants (Table 4).

3.7.4. Anticipated use of the NF as a food supplement

The applicant has proposed a maximum daily intake of 4.0 g 3-FL/day in FS for individuals above 3 years of age or at a maximum level of 2.0 g 3-FL/day for infants (0–11 months) and young children (12–35 months) (Table 7).

Table 7: Use of the NF in FS and resulting intake expressed as mg/kg bw per day

Population group	Age (years)	Body weight[(a)] (kg)	Use level 3-FL (g/day)	Intake of 3-FL (mg/kg bw per day)[(b)]
Infants	< 1	5.0	2.0	400
Young children[(c)]	1 to < 3	12.0	2.0	167
Other children	3 to < 10	23.1	4.0	173
Young adolescents	10 to < 14	43.4	4.0	92
Older adolescents	14 to < 18	61.3	4.0	65
Adults	≥ 18	70.0	4.0	57

NF: Novel food; FS: Food supplements.
(a) Default and average body weights for each population group are available in EFSA Scientific committee (2012).
(b) Intake in 'mg/kg bw per day' are calculated by considering the use levels in 'mg/d' and default body weights defined in EFSA Scientific Committee (2012).
(c) Referred as 'toddlers' in the EFSA food consumption comprehensive database (EFSA, 2011).

The Panel notes that the maximum dose of 2.0 g 3-FL/day in young children (body weight of 12 kg) and infants (body weight of 5 kg) results in a maximum intake of 167 and 400 mg 3-FL/kg bw, respectively (default body weight values from EFSA Scientific Committee (2012)). In addition, the maximum daily intake of the NF from its use in FS (i.e., 4.0 g 3-FL/day) results in a maximum daily intake ranging from 57 (adults) to 173 (other children) mg 3-FL/kg bw in the general population (Table 7).

The Panel notes that the maximum daily intake of 3-FL from the use of the NF in FS (i.e., 57–400 mg/kg bw per day) does not exceed the mean estimated highest daily intake of 3-FL of 460 mg/kg bw in breastfed infants (Table 4).

FS are not intended to be used if other foods with added 3-FL are also consumed on the same day. For infants and young children, FS are not intended to be used if human milk or other foods with added NF are consumed on the same day.

3.7.5. Combined intake from the NF and other sources

The Panel notes that 3-FL is already authorised for use in food categories other than those proposed for the NF under assessment (i.e., processed cereal-based food) or at use levels that are higher than those proposed (e.g., cereal bars). The combined daily intake of 3-FL from the authorised and proposed uses, for each population group from each EU dietary survey, is available in the Excel file annexed to this scientific opinion under the Supporting Information section.

It is noted that the combined intake of 3-FL from already authorised uses and the currently proposed uses is similar in infants (highest P95th intake, Table 8) and slightly higher in young children and other children than the estimated intake based on only the currently proposed uses and use levels (highest P95th intake, Table 6).

Table 8: Intake estimate resulting from the combined uses of 3-FL from both authorised and proposed food categories at the maximum use levels

Population group	Age (years)	Mean intake (mg/kg bw per day)		P95th intake (mg/kg bw per day)	
		Lowest[(a)]	Highest[(a)]	Lowest[(b)]	Highest[(b)]
Infants	< 1	51	196	180	392
Young children[(c)]	1 to < 3	54	124	128	228
Other children	3 to < 10	22	70	49	129
Adolescents	10 to < 18	5	27	18	58
Adults[(d)]	≥ 18	11	14	27	34

(a) Intakes are assessed for all EU dietary surveys available in the food comprehensive database on 03 June 2023. The lowest and the highest averages observed among all EU surveys are reported in these columns.
(b) Intakes are assessed for all EU dietary surveys available in the food comprehensive database on 3 June 2023. The lowest and the highest P95th observed among all EU surveys are reported in these columns (P95th based on less than 60 individuals are not considered).
(c) Referred as 'toddlers' in the EFSA food consumption comprehensive database (EFSA, 2011).
(d) Includes elderly, very elderly, pregnant and lactating women.

The Panel notes that the highest estimated 95th percentile daily intake from the combined exposure (i.e., 392 mg/kg bw) from the maximum authorised and proposed uses, is higher than the estimated intake from the authorised uses alone (i.e., 366 mg/kg bw; EFSA NDA Panel, 2022a), and below the mean highest estimate for 3-FL intake from human milk (i.e., 460 mg/kg bw; Table 4).

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

No ADME data have been provided for the NF.

The applicant reported the assessment performed by the NDA Panel on previously evaluated HiMOs (EFSA NDA Panel, 2020a) concluding that the NF does not undergo any significant digestion by human enzymes in the upper gastrointestinal tract and that only small amounts are expected to be absorbed. Milk oligosaccharides are then fermented in the colon by intestinal microbiota with a fraction excreted unchanged in the faeces and a small fraction found in the urine (EFSA NDA Panel, 2022a).

Finally, there are no indications that the absorption of 3-FL, which is the main constituent of the NF or other structurally related mono- and oligosaccharides (e.g., D-lactose) from the NF, differs from that of similar components found in human milk (EFSA NDA Panel, 2021).

3.9. Nutritional information

The NF is mainly composed of the non-digestible oligosaccharide 3-FL (about 95% w/w DM). The remaining constituents include D-lactose, 3-fucosyllactulose and L-fucose (see Section 3.4).

D-Lactose is the most prevalent molecule in human milk (~ 7 g/100 mL) and its monomers glucose and 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). 3-Fucosyllactulose is an isomer of 3-FL, arising from the isomerisation of the glucose moiety at the reducing end of 3-FL to fructose. This pH- and temperature-dependent isomerisation reaction, also known as the Lobry de Bruyn–van Ekenstein reaction (Angyal, 2001; Wang, 2010), has been commonly reported for the conversion of lactose into lactulose during heat treatment of milk, including human milk (Beach and Menzies, 1983; Schuster-Wolff-Bühring et al., 2010; Gómez de Segura et al., 2012). Although the isomerisation product of 3-FL has not been specifically evaluated in heat-treated human milk, lactulose has also been detected at significant proportions of lactose (Gómez de Segura et al., 2012), and it can thus be reasonably assumed that 3-Fucosyllactulose is present at comparable ratios and can thereby be equally regarded to have a history of safe use from heat-treated human milk.

The Panel considers that consumption of the NF at the proposed use levels is not nutritionally disadvantageous.

3.10. Toxicological information

The applicant provided three toxicological studies on the NF, which were conducted in compliance with Organisation for Economic Co-operation and Development (OECD) principles of Good Laboratory Practices (GLPs) (OECD, 1998) and in accordance with the OECD test guidelines TG No 471, 487, and 408. An additional preliminary *in vivo* repeated study was also carried out. These studies, which were claimed proprietary by the applicant, are listed in Table 9. A publication on the assessment of the NF which describes these studies is also available (Phipps et al., 2022).

In addition, the applicant made reference to toxicological information from studies conducted with the 2'-FL, the isomer of 3-FL, that was assessed previously by EFSA (EFSA NDA Panel, 2015a,b,c, 2019a).

Table 9: List of toxicological studies with the NF

Reference	Type of study	Test system	Dose
Study No. 8429603 (Phipps et al., 2022)	Bacterial reverse mutation test (GLP, OECD TG 471 (1997))	*Salmonella* Typhimurium TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA	Up to 5,000 μg 3-FL/plate (absence and presence of S9 mix)
Study No. 8436271 (Phipps et al., 2022)	*In vitro m*icronucleus test in human lymphocytes (GLP, OECD TG 487 (2016))	Human lymphocytes	500, 1,000 and 2,000 μg 3-FL/mL (absence and presence of S9 mix)
Study No. 8438088 (Phipps et al., 2022)	14-day DRF oral toxicity study	SD rats	3,000 and 4,000 mg 3-FL/kg bw per day
Study No. 8437221 (Phipps et al., 2022)	90-day repeated dose oral toxicity study with a 28-day recovery period (GLP, OECD TG 408 (2018))	SD rats	1,000, 2,000 and 4,000 mg 3-FL/kg bw per day

DRF: Dose range finding; GLP: Good Laboratory Practice; OECD: Organisation for Economic Co-operation and Development; SD: Sprague–Dawley.

3.10.1. Genotoxicity

The *in vitro* assessment of the mutagenic potential of the NF (unpublished study report, 2021a) was performed with *S.* Typhimurium strains TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA (pKM101), which were exposed to 3-FL in water at five different concentrations up to 5,000 μg/plate, either in the presence or absence of liver microsomal fractions (S9 mix), using the plate-incorporation and pre-incubation methods (main study). Based on the results of a preliminary test, concentrations of 3-FL at 5,000, 1,500, 500, 150 and 50 μg/plate were selected for the main test. No evidence of toxicity was obtained following exposure to the NF and no precipitate was observed in any plate. Treatment with the NF did not result in increases in the number of revertant colonies as compared to the negative control at any concentration in both tests either in the presence or absence of S9 mix. Therefore, 3-FL was shown to be non-mutagenic in the absence or presence of metabolic activation at concentrations up to 5,000 μg 3-FL/plate.

The genotoxic potential of the NF was further investigated in an *in vitro* mammalian cell micronucleus test (unpublished study report, 2021b) conducted in cultured human peripheral blood lymphocytes in the presence or absence of metabolic activation (S9 mix). Based on the results of a preliminary test, three concentrations of 3-FL of 500, 1,000 and 2,000 μg/mL were tested. No statistically significant increases in the number of binucleated cells containing micronuclei both after 3-h treatment in the presence or absence of S9 mix or following 20-h treatment in the absence of S9 mix as compared to the negative control were recorded. The NF was therefore determined to be non-clastogenic and non-aneugenic in the absence and presence of metabolic activation up to the highest concentration of 2,000 μg 3-FL/mL.

Taking into account the test results 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. Repeated dose toxicity studies

The applicant provided a 14-day repeated dose pilot toxicity study where two groups of 8 Crl:CD (SD) neonatal (from Day 7 of age) rats/sex were given 3,000 or 4,000 mg 3-FL/kg bw per day by oral gavage (unpublished study report, 2021c). The treatment was well tolerated, the only clinical sign observed was skin reddening in the perianal region in a few rats in both sexes at high dose in the first days of dosing. Yellow staining of the same area up to the end of dosing phase was noted in some rats of both sexes and dose levels. No clinical pathology investigations were carried out and no alterations at gross pathology examination were noted. The high dose of 4,000 mg 3-FL/kg bw per day was selected as the top dose to be used in the main 90-day study.

In the 90-day study groups of 10 Crl:CD(SD) neonatal (from day 7 of age) rats/sex were given by oral gavage the vehicle (sterile water), 1,000, 2,000 or 4,000 mg 3-FL/kg bw per day (unpublished study report, 2021d; Phipps et al., 2022). An additional group was given oligofructose as the reference control group. In the two control groups and in the high-dose groups, five additional rats/sex were added for a 4-week recovery period.

There were no deaths in the course of the study and no treatment-related changes in clinical signs, food consumption, body weight and body weight gain were observed in any rats. Episodes of reddening of the skin and yellow staining in the perianal region at the beginning of the treatment period were observed mainly in some rats given 4,000 mg/kg/day for both 3-FL and the reference control group. A statistically significant decrease in body weight gain and reduced body weight was noted in the first month of dosing at intermediate and high dose in both sexes (reference control group included) with normalisation thereafter. Sporadic episodes of decreased food consumption at the highest dose were also recorded. Overall, there were no effects of 3-FL on the eye opening and pre-weaning development tests, ulna length, neuro-behavioural and ophthalmoscopic examinations, age or body weight at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation or vaginal opening for males and females, respectively). Similar performances across groups in the functional observation battery tests were recorded.

No test item-related adverse effects on haematology, clinical chemistry, thyroid hormone analysis or urinalysis parameters were observed. Sporadic statistically significant changes considered of no toxicological relevance were recorded at the end of the treatment period (decreased activated partial thromboplastin time at blood chemistry; increased pH, reduced specific gravity and reduced concentrations of protein, creatinine and glucose in the urine). No differences in these parameters were observed at the end of the 4-week recovery period.

A few statistically significant differences were observed for organ weights (relative to body weight, lower brain weight for males given 2,000 or 4,000 mg/kg per day; increased liver weight for males given 1,000 mg/kg per day; lower kidney weights for females given 2,000 or 4,000 mg/kg per day) and considered by the Panel as not biologically relevant since of small magnitude, without a clear dose–response relationship, limited to only one sex and in absence of microscopic findings.

There were a few macroscopic or histopathological abnormalities recorded that were considered spontaneous and/or incidental because they occurred at a low incidence rate, were randomly distributed across groups (controls included), or were expected in rats of this age and strain.

The Panel considers that no adverse effects were observed in this study up to the highest tested dose of 4,000 mg 3-FL/kg bw per day.

3.10.3. Human data

No human intervention studies with the NF were provided by the applicant. Reference was made to studies conducted with the isomer 2'-FL, and that data were overall sufficient to conclude previously about the safety of its use under the proposed conditions (EFSA NDA Panel, 2015a,b,c).

The Panel noted that a double-blind, controlled, randomised interventional study was conducted in infants with IF containing a mixture of HiMOs (5.75 g/L, corresponding to about 0.9 g/L of 3-FL). The safety and tolerability profile of the HiMO mixture–IF appeared similar to the commercialised IF alone used as a comparator (Parschat et al., 2021; EFSA NDA Panel, 2022a,f,g).

The Panel considers the information as supportive for the assessment of 3-FL.

3.11. Allergenicity

The applicant did not identify an allergenic potential of introduced proteins as a result of the genetic modification of the *E. coli* K-12 DH1 parental strain, according to the 'Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed of the Scientific Panel on Genetically Modified Organisms' (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 (≤ 0.01% w/w) 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 3-FL, but it also contains D-lactose, L-fucose, 3-fucosyllactulose and a small fraction of other related saccharides. The NF is produced by fermentation by a genetically modified strain (*E. coli* K-12 DH1 MDO MAP1834) of *E. coli* K-12 DH1 (DSM 4235).

The applicant intends to add the NF to a variety of foods, including IF and FOF, FSMP and FS. The target population proposed by the applicant is the general population.

Considering that 3-FL is a naturally occurring oligosaccharide present in human milk, the history of human exposure to 3-FL concerns breastfed infants. The intake of 3-FL in breastfed infants on a bw basis is expected to be safe also for other population groups.

The Panel notes that safety assessments of 3-FL, when produced by genetically modified strains of *E. coli* K-12 MG1655 or *E. coli* BL21 (DE3), have been carried out by EFSA (EFSA NDA Panel, 2021, 2022a) and that 3-FL produced by the above-mentioned genetically modified strains is included in the Union list of authorised NFs. The Panel also notes that other HiMOs (2'-FL/DFL, LNT, 3'-SL and 6'-SL sodium salts) produced by fermentation by genetically modified strains of the same parental strain *E. coli* K-12 DH1 have been assessed with positive outcomes (EFSA NDA Panel, 2019a,b, 2020b,c).

The submitted toxicity studies did not raise safety concerns. The Panel considers that no adverse effects were observed in the subchronic toxicity study up to an intake of 4,000 mg 3-FL/kg bw per day.

The Panel notes that the anticipated daily intake of 3-FL from the consumption of IF (only), in infants up to 16 weeks of age, does not exceed the mean highest intake level of 3-FL in breastfed infants on a bw basis. The anticipated daily intake of 3-FL from both proposed and combined (authorised and proposed) uses at their respective maximum use levels in all population categories is also not above the mean highest intake level of 3-FL from human milk in infants on a bw basis.

The maximum daily intake of 3-FL in FS for individuals above 3 years of age (i.e., 4 g/day) or in infants and young children (i.e., 2 g/day) does not exceed the mean highest intake level of 3-FL in

breastfed infants per kg bw. The applicant stated that FS containing the NF are not intended to be used if other foods with added NF or human milk are consumed on the same day.

Taking into account the intrinsic nature of HMOs with their limited absorption, the absence of toxicologically relevant effects in the subchronic study and considering that infants are naturally exposed to these substances, the Panel considers that the consumption of the NF at the proposed uses and use levels does not raise safety concerns.

Finally, it is noted that, 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 by breastfed infants and the estimated intake as NF.

5. Conclusions

The Panel concludes that the NF, which is composed of 3-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, HPLC-ESI-MS/MS and HPAEC-PAD; (ii) detailed description of the production process; (iii) information on the genetically modified production strain; (iv) composition and stability of the NF; (v) toxicological information, including *in vitro* genotoxicity studies, 14-day repeated dose pilot toxicity study and 90-day subchronic toxicity study (Table 9).

6. Steps taken by EFSA

1) On 04 October 2021, EFSA received a letter from the EC with the request for a scientific opinion on the safety of microbiologically produced 3-FL as a NF. Ref. Ares (2021)6018277.
2) On 04 October 2021, a valid application on 3-FL, which was submitted by Glycom A/S, was made available to EFSA by the EC through the Commission e-submission portal (NF 2021/2463) and the scientific evaluation procedure was initiated.
3) On 18 March 2022, EFSA requested the applicant to provide additional information or to clarify previously submitted one, to accompany the application and the scientific evaluation was suspended.
4) On 31 March 2023, additional information was provided by the applicant through the Commission e-submission portal and the scientific evaluation was restarted.
5) During its meeting on 27 April 2023, the NDA Panel, having evaluated the data, adopted a scientific opinion on the safety of microbiologically produced 3-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
ACVA	4,4′-Azobis-(4-cyanovaleric acid)
ADME	Absorption, Distribution, Metabolism and Excretion
AFNOR	Association Francaise de Normalisation
BIOHAZ	EFSA Panel on Biological Hazards
BRD	Bacteriology Reference Department
bw	Body weight
CAS	Chemical Abstracts Service
CFU	Colony forming units
CID	Collision-induced dissociation
Crl:CD(SD) rats	Charles River Laboratories: Cesarean-derived (Sprague Dawley) rats
DEPT-Q	Distortionless enhancement by polarisation transfer with retention of quaternaries
DFL	Difucosyllactose
DM	Dry matter
DRF	Dose-range finding
DSMZ	German Collection of Microorganisms and Cell cultures
EN	European norm
EU	Endotoxin units
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
Gal	Galactose
Glc	Glucose
g-DQFCOSY	Gradient double-quantum-filtered correlation spectroscopy
g-HMBC	Gradient heteronuclear multiple bond coherence
g-HSQC	Gradient heteronuclear single quantum coherence
GLP	Good Laboratory Practices
GMO	EFSA Panel on Genetically Modified Organisms
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis Critical Control Points
HiMO	Human-identical milk oligosaccharide
HMO	Human milk oligosaccharide
HPAEC-PAD	High-performance anion-exchange chromatography – pulsed amperometric detection
HPLC-CAD	High-performance liquid chromatography – charged aerosol detection
HPLC-ESI	High-performance liquid chromatography – electrospray ionisation
ICP-MS	Inductively coupled plasma – mass spectrometry
IF	Infant formula
ISO	International Organization for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LNnT	Lacto-*N*-neotetraose
LNT	Lacto-*N*-tetraose
LOQ	Limit of quantification
m	Modification of analytical methods
MDO	Membrane-derived oligosaccharides
MS/MS	Tandem mass spectrometry
MSZ	Polish Ministry of Foreign Affairs
NANA	*N*-acetyl-D-neuraminic acid
ND	Not detected
NDA	EFSA Panel on Nutrition, Novel Foods and Food Allergens
NF	Novel food

NMKL	Nordisk Metodikkomite for Levnedsmidler
NMR	Nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
Ph. Eur.	European Pharmacopoeia
qPCR	Quantitative polymerase chain reaction
QPS	Qualified presumption of safety
RH	Relative humidity
rRNA	Ribosomal ribonucleic acid
SD rats	Sprague Dawley rats
TG	Test guidelines
UHT	Ultra-high temperature
US	United States
US EPA	United States Environmental Protection Agency
w/w	Weight per weight
ZKBS	Central Committee on Biological Safety

Appendix A – Authorised and proposed uses and use levels for 3-FL

EU Food category number	Food category	Proposed maximum use level (expressed as 3-FL)	Authorised maximum use levels (as included in the Union list Reg. (EU) 2017–2470)	
			'Microbial source'	'Produced by a derivative strain of *E. coli* BL21(DE3)'
1	**Dairy products and analogues**			
1.1	Unflavoured pasteurised and unflavoured sterilised (including UHT) milk	2.0 g/L	0.85 g/L	–
1.2/1.3	Unflavoured fermented milk-based products	2.0 g/L beverages	0.5 g/L beverages 5.0 g/kg products other than beverages	–
		4.0 g/kg products other than beverages		
1.4	Flavoured fermented milk-based products including heat-treated products	2.0 g/L beverages		
		12.0 g/kg products other than beverages		
7	**Bakery wares**			
7.2	Fine bakery wares. Cereal bars only	25.0 g/kg	30.0 g/kg	–
13	**Foods for Special Groups (FSG)**			
13.1	**Foods for infants and young children**			
13.1.1	Infant formula as defined in Regulation (EU) No 609/2013	1.75 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	0.85 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	0.90 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
13.1.2	Follow-on formula as defined in Regulation (EU) No 609/2013	1.75 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	0.85 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	1.20 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
13.1.3	Processed cereal-based food and baby food for infants and young children as defined under Regulation (EU) No 609/2013	–	0.3 g/L (beverages) in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	1.20 g/L or 1.20 g/kg in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
		–	3.0 g/kg for products other than beverages	–

EU Food category number	Food category	Proposed maximum use level (expressed as 3-FL)	Authorised maximum use levels (as included in the Union list Reg. (EU) 2017–2470)	
			'Microbial source'	'Produced by a derivative strain of *E. coli* BL21(DE3)'
13.1.4	Milk-based drinks and similar products intended for young children	2.0 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	0.85 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	1.20 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
		12 g/kg for products other than beverages	–	–
13.2	**Foods for special medical purposes as defined in Regulation (EU) No 609/2013**			
13.2	Foods for special medical purposes as defined in Regulation (EU) No 609/2013	On case-by-case basis	In accordance with the particular nutritional requirements of the infants and young children for whom the products are intended	–
	Foods for special medical purposes for infants and young children as defined under Regulation (EU) No 609/2013	–	–	In accordance with the particular nutritional requirements of the infants and young children for whom the products are intended but in any case not higher than 0.9 g/L or 0.9 g/kg (if it is intended for infants from 0 until 6 months) and 1.2 g/L or 1,2 g/kg (if it is intended for infants of 6–12 months and/or for young children) in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer.
	Foods for special medical purposes as defined under Regulation (EU) No 609/2013 excluding foods for infants and young children	–	–	In accordance with the particular nutritional requirements of the persons for whom the products are intended
13.3	**Total diet replacement for weight control as defined in Regulation (EU) No 609/2013**			
13.3	Total diet replacement for weight control as defined in Regulation (EU) No 609/2013	2.0 g/L	2.0 g/L	–
		25.0 g/kg for products other than beverages	30.0 g/kg for products other than beverages	–

EU Food category number	Food category	Proposed maximum use level (expressed as 3-FL)	Authorised maximum use levels (as included in the Union list Reg. (EU) 2017–2470)	
			'Microbial source'	'Produced by a derivative strain of *E. coli* BL21(DE3)'
14	**Beverages**			
14.1.4	Flavoured drinks (excluding cola-type drinks)	1.25 g/L	1.0 g/L	–
17	**Food supplements as defined in Directive 2002/46/EC**			
	Food supplements for infants and young children	2.0 g/day	Excluding food supplements for infants and young children	–
	Food supplements for ages over 3 years	4.0 g/day	5.0 g/day	–

Annex A – Dietary exposure estimates to the Novel Food for each population group from each EU dietary survey (proposed and combined uses and use levels)

Information provided in this Annex can be found in the online version of this output (in the 'Supporting information' section).