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Safety of 3′-sialyllactose (3′-SL) sodium salt produced by a derivative strain (*Escherichia coli* NEO3) of *E. coli* W (ATCC 9637) as a Novel Food pursuant to Regulation (EU) 2015/2283

EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), Dominique Turck, Torsten Bohn, Jacqueline Castenmiller, Stefaan De Henauw, Karen Ildico Hirsch-Ernst, Alexandre Maciuk, Inge Mangelsdorf, Harry J McArdle, Androniki Naska, Kristina Pentieva, Alfonso Siani, Frank Thies, Sophia Tsabouri, Marco Vinceti, Margarita Aguilera-Gómez, Francesco Cubadda, Thomas Frenzel, Marina Heinonen, Rosangela Marchelli, Monika Neuhäuser-Berthold, Carmen Peláez, Morten Poulsen, Miguel Prieto Maradona, Josef Rudolf Schlatter, Alexandros Siskos, Henk van Loveren, Paolo Colombo, Estefanía Noriega Fernández and Helle Katrine Knutsen

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′-sialyllactose (3′-SL) sodium salt as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The NF is mainly composed of the human-identical milk oligosaccharide (HiMO) 3′-SL (sodium salt), but it also contains sialic acid, D-glucose, D-lactose, 3′-sialyllactulose and 6′-sialyllactose sodium salts and a small fraction of other related saccharides. The NF is produced by fermentation by a genetically modified strain (*Escherichia coli* NEO3) of *E. coli* W (ATCC 9637). The information provided on the identity, 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 applicant applies for the same uses and use levels as already assessed for 3′-SL sodium salt produced by a genetically modified strain of *E. coli* K-12 DH1, with the exception for the use in FS, which is proposed to be higher (from 0.5 to 1.0 g/day) in individuals from 3 years of age. Since the NF as a food ingredient would be consumed at the same extent as the already assessed 3′-SL sodium salt, no new estimates of the intakes have been carried out. The Panel notes that the maximum daily intake of 3′-SL from the proposed use of the NF in FS for individuals from 3 years of age (1.0 g/day) is lower than the estimated highest mean daily intake of 3′-SL in breastfed infants. FS are not intended to be used if other sources of 3′-SL are consumed on the same day. The Panel concludes that the NF is safe under the proposed conditions of use.

Keywords: 3′-sialyllactose, 3′-SL, sodium salt, human milk oligosaccharide, HMO, HiMO, novel food, safety

Requestor: European Commission

Question number: EFSA-Q-2021-00445

Correspondence: nda@efsa.europa.eu

Panel members: Dominique Turck, Torsten Bohn, Jacqueline Castenmiller, Stefaan De Henauw, Karen Ildico Hirsch-Ernst, Helle Katrine Knutsen, Alexandre Maciuk, Inge Mangelsdorf, Harry J McArdle, Androniki Naska, Kristina Pentieva, Alfonso Siani, Frank Thies, Sophia Tsabouri and Marco Vinceti.

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1. Introduction

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

On 25 March 2021, the company Kyowa Hakko Bio Co., Ltd. submitted a request to the Commission in accordance with Article 10 of Regulation (EU) 2015/2283[1] to place on the EU market 3′-sialyllactose (3′-SL) sodium salt as a novel food (NF).

3′-SL sodium salt is intended to be used in a number of food categories.

The applicant has requested data protection under Article 26 of Regulation (EU) 2015/2283 for data in support of this request.

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′-SL sodium salt as a NF.

In this opinion on 3′-SL sodium salt, EFSA should also document whether and to what extent the requirements of Article 26(2)(c) of Regulation (EU) 2015/2283 are fulfilled regarding the data for which the applicant is requesting data protection.

1.2. Additional information

Sodium salts of 3′-SL produced by fermentation by genetically modified strains of *Escherichia coli* K-12 DH1 or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2020a, 2022a) are included in the Union list of authorised NFs (Commission Implementing Regulation (EU) 2017/2470[2]). The sodium salt of 6′-sialyllactose (6′-SL), a constitutional isomer of 3′-SL, is also included in the Union list of authorised NFs when produced by fermentation by genetically modified strains of *E. coli* K-12 DH1 or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2020b, 2022b). Moreover, the safety of 6′-SL sodium salt produced by *E. coli* NEO6, a genetically modified strain of the same parental strain *E. coli* W (ATCC 9637), has been assessed by EFSA with a positive outcome (EFSA NDA Panel, 2023a).

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 synthetised 2′-fucosyllactose (2′-FL) (EFSA NDA Panel, 2015a) and 2′-FL produced by a genetically modified strain (APC199) of *Corynebacterium glutamicum* ATCC 13032 (EFSA NDA Panel, 2022c);
- Chemically synthetised lacto-N-neotetraose (LNnT) (EFSA NDA Panel, 2015b) and LNnT produced by genetically modified strains of *E. coli* BL21 (DE3) (EFSA NDA Panel, 2020c);
- Extension of use in food supplements (FS) for children of chemically synthetised 2′-FL and LNnT (EFSA NDA Panel, 2015c) and extension of use in FS for infants of 2′-FL and LNnT produced by genetically modified strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2022d);
- Chemically synthetised N-acetyl-D-neuraminic acid (NANA) (EFSA NDA Panel, 2017);
- 2′-FL/difucosyllactose (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, 2022f);
- 3′-SL sodium salts produced by genetically modified strains of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020a) or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022a);
- 6′-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, 2022b), or by *E. coli* NEO6, a genetically modified strain of the same parental strain *E. coli* W (ATCC 9637) (EFSA NDA Panel, 2023a);

[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, 30.12.2017, pp. 72–201.

- 3-fucosyllactose (3-FL) produced by genetically modified strains of *E. coli* K-12 MG1655 (EFSA NDA Panel, 2021), *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022g) or *E. coli* K-12 DH1 (EFSA NDA Panel, 2023b).

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) toxicological and allergenicity studies.

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 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 the 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 Directive92/52/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, p. 35–56.

[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, p. 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, p. 1–29.

3. Assessment

3.1. Introduction

The NF, which is the subject of the application, contains 3′-SL sodium salt as primary constituent (≥ 82.0% w/w dry matter (DM)). 3′-SL has been identified as a relevant component of the complex fraction of oligosaccharides naturally occurring in human milk, also denominated as human milk oligosaccharides (HMOs). 3′-SL is a sialylated (acidic) trisaccharide composed of D-glucose, D-galactose and NANA (hereinafter also referred to as 'sialic acid'). The Panel notes that although the 3′-SL sodium salt is the major component of the NF, related substances, namely sialic acid, D-glucose, D-lactose, 3′-sialyllactulose and 6′-SL sodium salts and a small fraction of other related saccharides, are also present. The NF is produced by fermentation by a genetically modified strain (*E. coli* NEO3) of *E. coli* W (ATCC 9637) and is isolated as a purified ingredient in the sodium salt form.

The applicant applies for the same uses and use levels (as a food ingredient, including FSMP, IF, FOF and FS) already assessed for the 3′-SL sodium salt produced by fermentation by a genetically modified strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020a). In addition, the applicant has requested a higher maximum use level, from 0.5 to 1.0 g/day, in FS for individuals from 3 years of age.

The target population is the general population.

Sodium salts of 3′-SL produced by genetically modified strains of *E. coli* K-12 DH1 or *E. coli* BL21 (DE3) (EFSA NDA Panel, 2020a, 2022a) are already 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′-SL sodium salt (≥ 82.0% w/w DM), but it also contains sialic acid (≤ 6.0% w/w DM), D-glucose (≤ 3.0% w/w DM), D-lactose (≤ 3.0% w/w DM), 3′-sialyllactulose and 6′-SL sodium salts (≤ 5.0% w/w DM, sum of both), and a small fraction of other related saccharides (sum of other carbohydrates ≤ 12.0% w/w DM). It is produced by fermentation by a genetically modified strain (*E. coli* NEO3) of *E. coli* W (ATCC 9637). The main component is the sodium salt of Neu5Ac-α-(2–3)-Gal-β-(1–4)-Glc (3′-SL), in which sodium N-acetyl-D-neuraminate is linked through an α-(2–3) bond to D-galactose, which is linked through a β-(1–4) bond to D-glucose, in its α- and β-anomeric forms (Table 1 and Figure 1). 3′-SL is a regioisomer of 6′-SL, which contains the same monosaccharide moieties as those present in 3′-SL but with the linkage between N-acetyl-D-neuraminic acid (Neu5Ac) and D-galactose being α-(2–6) instead of α-(2–3).

Table 1: Chemical identity of 3′-SL sodium salt

Chemical substance	
Chemical (IUPAC) name	Sodium; (2*S*,4*S*,5*R*,6*R*)-5-acetamido-2-[(2*R*,3*S*,4*S*,5*R*,6*S*)-3,5-dihydroxy-2-(hydroxymethyl)-6-[(2*R*,3*S*,4*R*,5*R*)-4,5,6-trihydroxy-2-(hydroxymethyl)oxan-3-yl] oxyoxan-4-yl]oxy-4-hydroxy-6-[(1*R*,2*R*)-1,2,3-trihydroxypropyl]oxane-2-carboxylate
Common name	3′-Sialyllactose, sodium salt
Abbreviations	3′-SL, sodium salt
Alternative chemical names	• N-Acetyl-α-D-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose, sodium salt • 3′-SL sodium salt • 3′-N-acetylneuraminyl-D-lactose sodium salt • α-D-Neu5Ac-(2→3)-β-D-Gal-(1→4)-D-Glc sodium salt
CAS number	128596-80-5 (sodium salt)/35890-38-1 (acid)
Molecular formula	$C_{23}H_{38}NO_{19}Na$
Molecular mass	655.53 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′-SL, the major constituent of the NF.

The structure of 3′-SL[8] was determined by mono-dimensional (1D) nuclear magnetic resonance (NMR) spectroscopy, including ^{1}H and ^{13}C spectra, and two-dimensional (2D) NMR spectroscopy, including COSY (correlation spectroscopy), TOCSY (total correlation spectroscopy), HETCOR (heteronuclear correlation) and HMBC (heteronuclear multiple bond correlation) spectra, by comparison to a commercially available authentic specimen.[9] The relevant coupling constants measured by ^{1}H NMR together with the correlations evidenced on the 2D NMR spectra confirmed: (i) the α-(2″-3′) glycosidic linkage between Neu5Ac (C-2″) and the D-galactose (Gal-C-3′) moiety of D-lactose; (ii) the β-(1′-4) link between the D-galactose (Gal-C-1′) and D-glucose (Glc-C-4) moieties of D-lactose; and (iii) the β configuration of the Gal unit.

The molecular structure of 3′-SL[8] was corroborated by liquid chromatography – tandem mass spectrometry (LC–MS/MS) based on its retention factor (R_f) and fragmentation pattern, by comparison to a commercially available high-purity analytical standard, which allowed to differentiate between 3′-SL α-(2″-3′) and 6′-SL α-(2″-6′).

The identity of 3′-SL[8] was also corroborated by high-performance liquid chromatography – charged aerosol detection (HPLC-CAD) by comparison to a commercially available high-purity analytical standard.

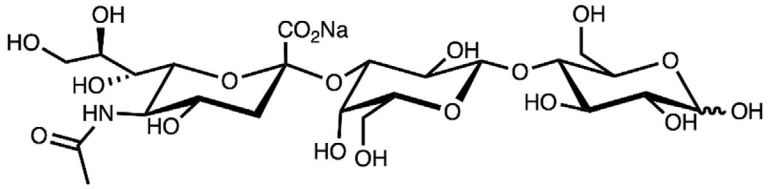

Figure 1: Chemical structure of 3′-SL sodium salt (EFSA NDA Panel, 2022a)

On the basis of the spectroscopic and chromatographic evidence, the Panel considers that the 3′-SL present in the NF produced by *E. coli* NEO3 is identical to the 3′-SL 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, in a facility that is FSSC (Food Safety System Certification) 22000 certified.

The NF is produced by fermentation by a genetically modified strain (*E. coli* NEO3) of *E. coli* W (ATCC 9637) using food-grade raw materials and processing aids. The production microorganism is cultured under sterile conditions in a chemically defined nutrient medium (without soy peptone for commercial production purposes) and uses glucose and lactose to synthesise 3′-SL, which is excreted into the medium. The production microorganism is removed from the culture medium by microfiltration at the end of the fermentation process. 3′-SL is isolated as the sodium salt and purified from the fermentation medium using a series of filtration and cationic and anionic exchange chromatography steps, followed by concentration and spray-drying to obtain the final 3′-SL sodium salt product in powder form.

The production strain *E. coli* NEO3 is a genetically modified derivative of the parental strain *E. coli* W (Waksman's strain), which is deposited at the American Type Culture Collection (ATCC) (commercially available under ATCC 9637). The strain *E. coli* W is well-characterised and its genome has been sequenced, annotated and compared to other safe *E. coli* strains and phylogroup B1 commensal/pathogenic *E. coli* strains (Archer et al., 2011). Although *E. coli* W harbours genes that encode pathogenicity determinants, these have been mutationally inactivated or are missing key components required for pathogenicity, similarly to other safe strains (Archer et al., 2011). Genomic analyses also confirmed the lack of genes encoding toxins that can be secreted (Archer et al., 2011). Although the species *E. coli* is considered not suitable for qualified presumption of safety (QPS) status (EFSA BIOHAZ Panel, 2023), the strain *E. coli* W does not cause disease in healthy adult humans, does

[8] Batches of the NF produced with and without soy peptone in the fermentation media.
[9] 3′-SL isolated from human milk.

not colonise the human gut (Bauer et al., 2008; NIH, 2019), and is considered as a safe, non-pathogenic and non-toxigenic microorganism widely used for biotechnological applications.

The production strain has been deposited at the Japanese National Biological Resource Center (NBRC) culture collection. A detailed description of the genetic modification steps applied to the parental strain *E. coli* W to obtain the production strain *E. coli* NEO3 has been provided by the applicant. No residual DNA from the production strain was detected in the NF by a quantitative polymerase chain reaction (qPCR) assay using primers specific to the production strain. 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 characteristics, the applicant provided analytical information for eight batches of the NF produced with (five batches) or without (three batches) soy peptone in the fermentation media, the latter representing the conditions for commercial production of the NF (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′-SL sodium salt as main component (93.0% w/w DM[10] / 88.0% w/w DM[11] in batches produced with / without soy peptone in the fermentation media, respectively). The remaining constituents[12] include sialic acid (3.3% w/w DM[10] / 3.1% w/w DM[11]), D-lactose (≤ 0.08% w/w DM[10] / < 0.05% w/w DM[11]), D-glucose (< 0.02% w/w DM[10] / < 0.05% w/w DM[11]), 3′-sialyllactulose and 6′-SL sodium salts (0.4% w/w DM[10–11] – sum of both carbohydrates), and a small fraction of other related saccharides (sum of other carbohydrates[13], 2.3% w/w DM[10] / 7.2% w/w DM[11]).

With regards 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 produced with soy peptone and three batches without soy peptone in the fermentation media, 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 427 g/L and 410 g/L, respectively.

[10] Average content in five batches of the NF produced with soy peptone in the fermentation media.
[11] Average content in three batches of the NF produced without soy peptone in the fermentation media.
[12] For those batches of the NF where the levels of any carbohydrate by-product were below the respective limit of quantification (LOQ) or limit of detection (LOD), the concentration of the corresponding compound has been considered to be equal to the respective LOQ or LOD value for the purpose of calculating its average content.
[13] Sum of other carbohydrates = 100% w/w DM – 3′-SL (acid) (% w/w DM) – quantified carbohydrates (i.e., sialic acid, D-glucose, D-lactose, 3′-sialyllactulose (acid) and 6′-SL (acid); % w/w DM) – sodium (% w/w DM).

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

Parameters	NF (produced with soy peptone in the fermentation media)					NF (produced without soy peptone in the fermentation media)			Method of analysis
	#1	#2	#3	#4	#5	#6	#7	#8	
Composition									
3′-SL sodium salt (% w/w DM)	89.0	94.0	93.0	95.0	94.0	82.0	91.0	91.0	HPLC-CAD (validated internal method)
Sialic acid (% w/w DM)	5.0	2.0	2.8	2.8	3.9	8.4*	0.5	0.4	HPLC-CAD[(a)] (validated internal method)
D-Glucose (% w/w DM)	< 0.02	< 0.02	< 0.02	< 0.02	< 0.02	< 0.05	< 0.05	< 0.05	HPLC-PAD[(b)] (validated internal method)
D-Lactose (% w/w DM)	0.1	≤ 0.05	0.1	≤ 0.05	0.1	< 0.05	< 0.05	< 0.05	HPLC-PAD[(b)] (validated internal method)
Sum of 3′-sialyllactulose and 6′-SL sodium salts (% w/w DM)	0.5	0.4	0.4	0.4	0.5	0.7	0.2	0.2	HPLC-CAD[(a),(c)] (validated internal method)
Sum of other carbohydrates (% w/w DM)	4.2	2.4	2.9	1.0	0.9	6.9	7.5	7.2	Calculation[(d)]
Water (% w/w)	5.4	5.2	5.0	5.5	5.7	7.2	8.2	8.2	JP 2.48[(e)] – Karl Fischer titration (volumetric/coulometric titration)
Ash (% w/w DM)	11.9	–	11.7	–	–	12.3	11.1	11.5	JP 2.44[(e)] (residue on ignition, gravimetry)
Protein (% w/w)	≤ 0.01	≤ 0.01	≤ 0.01	–	≤ 0.01	≤ 0.01	≤ 0.01	≤ 0.01	Bradford assay[(f)] (spectrophotometry)
Sodium (% w/w DM)	4.0	4.3	3.9	3.9	3.8	4.0	3.4	3.8	USP 233[(g)] (ICP-MS or ICP-OES)
pH (5% solution, 25°C)	6.5	6.5	6.4	6.3	6.4	5.9	5.7	5.8	JP 2.54[(e)] (potentiometry)
Contaminants									
Arsenic (mg/kg)	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	< 0.01[(i)]	< 0.01[(i)]	< 0.01[(i)]	USP 233[(g),(h)] (ICP-MS) AOAC (2019) 999.10 and 2011.14[(i)] (AAS and ICP-OES)
Cadmium (mg/kg)	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	< 0.01[(j)]	< 0.01[(j)]	< 0.01[(j)]	USP 233[(g),(h)] (ICP-MS) AOAC (2019) 999.10 and 2011.14[(j)] (AAS and ICP-OES)
Lead (mg/kg)	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	< 0.02[(k)]	< 0.02[(k)]	< 0.02[(k)]	USP 233[(g),(h)] (ICP-MS) AOAC (2019) 999.10 and 2011.14[(k)] (AAS and ICP-OES)
Mercury (mg/kg)	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	≤ 0.05	< 0.004[(l)]	< 0.004[(l)]	< 0.004[(l)]	USP 233[(g),(h)] (ICP-MS) US EPA, February 2007, Method 7473[(l)] (AAS)
Aflatoxin M1 (μg/kg)	< 0.02	–	< 0.02	–	< 0.02	< 0.02	< 0.02	< 0.02	AOAC 2000.08[(m)] (HPLC)

Parameters	NF (produced with soy peptone in the fermentation media)					NF (produced without soy peptone in the fermentation media)			Method of analysis
	#1	#2	#3	#4	#5	#6	#7	#8	
Microbial parameters									
Total plate count (CFU/g)	< 40	< 10	< 10	< 10	< 10	< 10	270	< 10	ISO 4833-1:2013[(n)] (colony count)
Yeasts and moulds (CFU/g)	< 100	< 100	< 100	< 100	< 100	< 10[(o)]	< 10[(o)]	< 10[(o)]	ISO 21527-2:2008[(p)] (colony count)
Enterobacteriaceae (in 10 g)	ND	ND	ND	ND	ND	ND	ND	ND	ISO 21528-1:2017 (detection or qualitative method)
Salmonella spp. (in 25 g)	ND	–	ND	–	ND	ND	ND	ND	ISO 6579-1:2017 (detection or qualitative method)
Cronobacter spp. (in 10 g)	ND	ND	ND	ND	ND	ND	ND	ND	ISO 22964:2017 (detection or qualitative method)
Listeria monocytogenes (in 25 g)	ND	ND	ND	ND	ND	ND	ND	ND	ISO 11290-1:2017 (detection or qualitative method)
Presumptive *Bacillus cereus* (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	ISO 7932:2004[(q)] (colony count)
Endotoxins (EU/mg)	0.044	0.006	0.036	0.011	0.019	< 0.0002[(r)]	< 0.0002[(r)]	< 0.0002[(r)]	JP17 4.01[(e)] (kinetic-turbidimetric method)

'–': Not reported; 3′-SL: 3′-Sialyllactose; 6′-SL: 6′-Sialyllactose; AAS: Atomic absorption spectroscopy; AOAC: Association of Official Analytical Collaboration; CFU: Colony forming units; DM: Dry matter; EU: Endotoxin units; HPLC-CAD: High-performance liquid chromatography – charged aerosol detection; HPLC-PAD: High-performance liquid chromatograph – pulsed amperometric detection; ICP-MS: Inductively coupled plasma – mass spectrometry; ICP-OES: Inductively coupled plasma – optical emission spectroscopy; ISO: International Organisation for Standardisation; JP: Japanese Pharmacopoeia; LOD: Limit of detection; LOQ: Limit of quantification; ND: Not detected; US EPA: United States Environmental Protection Agency; USP: United States Pharmacopeia; w/w: Weight per weight.

*Batch #6 to be discarded, as indicated by the applicant, since specifications for sialic acid are not met.

(a): For batches #1 to #5, the LOD and LOQ for sialic acid, 3′-sialyllactulose and 6′-SL sodium salts are, respectively, 0.01% w/w DM and 0.2% w/w DM (as 3′-SL sodium salt). For batches #6 to #8, the LOD and LOQ for sialic acid are, respectively, 0.28% w/w DM and 0.45% w/w DM (as sialic acid); the LOD and LOQ for 3′-sialyllactulose + 6′-SL sodium salts (sum of both) are, respectively, 0.11% w/w DM and 0.18% w/w DM (as 3′-SL sodium salt).

(b): For batches #1 to #5, the LOD for D-glucose and D-lactose is 0.02% w/w DM, and their LOQ is 0.05% w/w DM (as D-lactose). For batches #6 to #8, the LOD for D-glucose and D-lactose is 0.03% w/w DM, and their LOQ is 0.05% w/w DM (as D-glucose and D-lactose).

(c): 3′-sialyllactulose and 6′-SL peaks on the HPLC-CAD chromatograms overlap.

(d): Sum of other carbohydrates = 100% w/w DM – 3′-SL (acid) (% w/w DM) – quantified carbohydrates (i.e., sialic acid, D-glucose, D-lactose, 3′-sialyllactulose (acid) and 6′-SL (acid); % w/w DM) – sodium (% w/w DM). Concentrations in acid form for the respective carbohydrates have been theoretically calculated.

(e): Consistent with the compendial method specified in the 17th edition of the Japanese Pharmacopoeia (2016).

(f): Evaluated using a limit test at 0.01% w/w.

(g): Method is consistent with the compendial method specified in the United States Pharmacopeia 35th revision (2011). Batches #1 to #5 were analysed by ICP-MS (LOQ = 1.25%). Batches #6 to #8 were analysed by ICP-OES (LOQ = 0.005%).

(h): LOQ for arsenic, cadmium, lead and mercury = 0.05 mg/kg.

(i): Method based on AOAC (2019) 999.10 and 2011.14. LOD = 0.01 mg/kg. LOQ = 0.03 mg/kg.

(j): Method based on AOAC (2019) 999.10 and 2011.14. LOD = 0.01 mg/kg. LOQ = 0.03 mg/kg.

(k): Method based on AOAC (2019) 999.10 and 2011.14. LOD = 0.02 mg/kg. LOQ = 0.03 mg/kg.

(l): Method based on U.S. EPA, February 2007, Method 7473, Mercury Analyzer. LOD = 0.004 mg/kg. LOQ = 0.01 mg/kg.
(m): LOQ = 0.02 μg/kg.
(n): LOD = 10 CFU/g. In accordance with ISO 4833-1:2013, the presence of one to three colonies should be reported as < 40 CFU/g.
(o): LOD = 10 CFU/g (in-depth plating).
(p): LOD = 100 CFU/g (surface plating).
(q): LOD = 10 CFU/g.
(r): LOQ = 0.0002 EU/mg.

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 provided interim results for a 3-year (real-time) stability study at 25°C and 60% relative humidity (RH) on one batch of the NF produced with soy peptone in the fermentation media, including measurements of 3′-SL sodium salt, carbohydrate, sodium and water content, and physico-chemical parameters (pH, appearance, colour) up to 30 months and water activity measurements after 6 and 12 months. In addition, a 6-month accelerated stability study at 40°C and 75% RH was conducted on five batches of the NF produced with soy peptone in the fermentation media, including the above-mentioned parameters (water activity measured after 6 months of storage). Microbial parameters were also monitored after 24 months under normal storage conditions.

No significant changes in 3′-SL sodium salt, carbohydrate, sodium and water content, physico-chemical parameters and water activity were observed over the storage period under normal and accelerated conditions. Microbial parameters were also below the respective limits of detection after 24 months of storage under normal conditions. The applicant proposed a 30-month shelf-life under ambient conditions for the NF.

The Panel considers that, since the presence of soy peptone in the fermentation medium does not change the composition and stability of the NF substantially, 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

No stability data for the 3′-SL sodium salt in food matrices were provided.

The applicant referred to (i) the stability studies included in the GRAS (Generally Recognised As Safe) notification GRN (GRAS Notice) 766 (US FDA, 2019) on 3′-SL sodium salt in powdered IF (at room temperature for 24 months), milk (at 4 and 25°C for 45 days) and yoghurt (at 4°C for 45 days) and (ii) the stability of related HiMOs, e.g. 2′-FL, 2′-FL/DFL mixture, LNnT and sialic acid, in IF, FOF, yoghurt, ready-to-drink flavoured milk, citrus fruit drinks and cereal bars (EFSA NDA Panel, 2015a,b, 2017, 2019a).

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′-sialyllactose (3′-SL) sodium salt is a white to off-white powder produced by microbial fermentation and further isolated, purified and concentrated.	
Source: A genetically modified strain (*Escherichia coli* NEO3) of *E. coli* W (ATCC 9637).	
Parameter	**Specification**
Composition	
3′-SL sodium salt (% w/w DM)	≥ 82.0
Sialic acid (% w/w DM)	≤ 6.0
D-Lactose (% w/w DM)	≤ 3.0
D-Glucose (% w/w DM)	≤ 3.0
Sum of 3′-sialyllactulose and 6′-SL sodium salts[(1)] (% w/w DM)	≤ 5.0
Sum of other carbohydrates[(2)] (% w/w DM)	≤ 12.0
Water (% w/w)	≤ 10.5
Protein (% w/w)	≤ 0.01
pH (5% solution, 25°C)	4.5–7.5
Sodium (% w/w DM)	≤ 5.0

Contaminants	
Arsenic (mg/kg)	≤ 0.2
Lead (mg/kg)	≤ 0.2
Cadmium (mg/kg)	≤ 0.2
Mercury (mg/kg)	≤ 0.1
Aflatoxin M1 (μg/kg)	≤ 0.025
Microbiological parameters	
Total plate count (CFU/g)	≤ 1,000
Yeasts and moulds (CFU/g)	≤ 100
Enterobacteriaceae (in 10 g)	ND
Salmonella (in 25 g)	ND
Cronobacter spp. (in 10 g)	ND
Listeria monocytogenes (in 25 g)	ND
Presumptive *Bacillus cereus* (CFU/g)	≤ 50
Endotoxins (EU/mg)	≤ 10

3′-SL: 3′-Sialyllactose; 6′-SL: 6′-Sialyllactose; CFU: Colony forming units; DM: Dry matter; EU: Endotoxin units; ND: Not detected; w/w: Weight per weight.
(1): 3′-Sialyllactulose and 6′-SL peaks on the HPLC-CAD chromatograms overlap.
(2): Sum of other carbohydrates = 100% w/w DM – 3′-SL (acid) (% w/w DM) – quantified carbohydrates (i.e., sialic acid, D-glucose, D-lactose, 3′-sialyllactulose (acid) and 6′-SL (acid); % w/w DM) – sodium (% w/w DM).

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′-SL sodium salt, which is the major constituent of the NF, has been authorised as a NF in the EU (Commission Implementing Regulation (EU) 2021/96[14]) to be added to IF and FOF, to a variety of foods as well as to FS. The authorised 3′-SL sodium salt is produced by fermentation by a genetically modified strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020a). Another 3′-SL sodium salt produced by genetically modified strains of *E. coli* BL21 (DE3) has recently been assessed with a positive outcome (EFSA NDA Panel, 2022a; Commission Implementing Regulation (EU) 2023/113[15]).

3′-SL has also been detected in domestic farm animal milk, albeit generally at lower concentrations as compared to human milk. Oligosaccharides in bovine milk are 20 times less concentrated than in human milk; however, sialylated oligosaccharides account for approximately up to 80% of the total oligosaccharide pools. 3′-SL is the most represented oligosaccharide in bovine milk and its concentration is estimated to be ranging from 47 to 55 mg/L and over 1 g/L in bovine colostrum (Aldredge et al., 2013; Urashima et al., 2013; Albrecht et al., 2014). This is approximately six times lower than the respective concentration in human milk (EFSA NDA Panel, 2020a).

3.7. Proposed uses and use levels and anticipated intake

The applicant applies for the same uses and use levels already assessed for 3′-SL sodium salt produced by fermentation by a genetically modified strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020a). Therefore, since the NF would be consumed at the same extent as the already assessed 3′-SL sodium salt, no new estimates of the intake have been carried out.

However, the applicant proposes a higher maximum daily intake of 3′-SL sodium salt in FS for individuals from 3 years of age from 0.5 to 1.0 g/day.

[14] Commission Implementing Regulation (EU) 2021/96 of 28 January 2021 authorising the placing on the market of 3′-sialyllactose sodium salt 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 31, 29.1.2021, p. 201–207.
[15] Commission Implementing Regulation (EU) 2023/113 of 16 January 2023 authorising the placing on the market of 3′-sialyllactose sodium salt produced by derivative strain of *Escherichia coli* BL21(DE3) as a novel and amending Implementing Regulation (EU) 2017/2470, OJ L 15, 17.1.2023, p. 1–8.

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 intended uses and use levels proposed for the NF as a food ingredient are the ones already assessed for 3′-SL sodium salt manufactured by fermentation by a genetically modified strain of *E. coli* K-12 DH1. Selected food categories include: unflavoured milk products; fermented milk-based products, flavoured and unflavoured; beverages; cereal bars; infant and follow-on formulae; processed cereal-based food and baby foods; milk based drinks and similar products and total diet replacement foods for weight control.[2]

As a FS, the applicant proposes a higher maximum daily intake in individuals from 3 years of age from 0.5 g/day (as proposed for 3′-SL produced by a genetically modified strain of *E. coli* K-12 DH1) to 1.0 g/day.

3.7.3. Anticipated intake of the NF

Considering that the same uses and use levels as food ingredient as per the assessed 3′-SL are proposed (EFSA NDA Panel, 2020a), the Panel considers that a new assessment of the intake is not needed.

The NF would be used at the same extent as the authorised 3′-SL, with the exception for the use in FS in individuals from 3 years of age.

Anticipated intake of the NF from the use in food supplements

The applicant has proposed a maximum daily intake of 1.0 g when intended for individuals from 3 years of age, instead of the 0.5 g/day as authorised for 3′-SL produced by a genetically modified strain of *E. coli* K-12 DH1. The Panel notes that a maximum daily intake of 0.7 g was authorised for 3′-SL when produced by genetically modified strains of *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022; Commission Implementing Regulation (EU) 2023/113[15]).

Table 4: 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 (g/day)	Intake (mg/kg bw per day)[(b)]
Other children	3 to <10	23.1	1.0	43
Young adolescents	10 to <14	43.4	1.0	23
Older adolescents	14 to <18	61.3	1.0	16
Adults	≥ 18	70.0	1.0	14

bw: Body weight.
(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/day' and default body weights defined in EFSA Scientific Committee (2012).

The Panel notes that the maximum daily intake of 3′-SL from the use of the NF in FS (ranging from 14 to 43 mg/kg bw) for individuals from 3 years of age is lower than the estimated high daily intake of 3′-SL of 125 mg/kg bw in breastfed infants (EFSA NDA Panel, 2022a).

According to the applicant, FS containing the NF are not intended to be used if other sources of 3′-SL are consumed on the same day.

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

No ADME data were provided for the NF.

The applicant made reference to the assessment performed by the NDA Panel on a previously evaluated 3′-SL sodium salt (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 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).

In addition, cross reference is made to a study conducted with labelled 3′-SL (^{13}C-3′-SL and also its component ^{13}C-sialic acid) in mice (Galuska et al., 2020). The main goal of the study was to verify the ^{13}C enrichment in different areas of the brain. The study also demonstrated that absorption after oral administration occurs and is followed by rapid urinary excretion.

Finally, there are no indications that the absorption of 3′-SL, or other structurally related mono- and oligosaccharides (e.g. sialic acid) from the NF, differs from that of similar components in human milk.

3.9. Nutritional information

The NF is mainly composed of the non-digestible oligosaccharide 3′-SL.

The NF contains other carbohydrates, individually present at low concentrations. Sialic acid is an endogenous human and ubiquitous monosaccharide (EFSA NDA Panel, 2017; Röhrig et al., 2017). D-Lactose is the most abundant component of human milk (~ 7%) and its monomers, D-glucose and D-galactose, are normal constituents of human milk. 6′-SL is a regioisomer of 3′-SL and also a normal constituent of human milk (see Section 3.4).

The Panel notes that the NF, being a sodium salt, may contribute to the daily sodium intake. Since the same uses and use levels as per the authorised 3′-SL sodium salt are proposed and the sodium content is comparable, similar considerations for the sodium intake apply (i.e. intake ranging from 2% to 14% of the safe and adequate intake[16] in different population groups; EFSA NDA Panel, 2020a).

With the higher maximum daily intake in FS, the sodium intake might reach a value of about 4% of the safe and adequate intake of 1.3 g/day in 'other children' (EFSA NDA Panel, 2019c) and lower values in other population groups.

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 applicant provided four toxicological studies on the NF, which were conducted in compliance with OECD principles of GLP (Organisation for Economic Co-operation and Development principles of Good Laboratory Practices (OECD, 1998)) and in accordance with the OECD test guidelines TG No 471, 474, 487 and 408. These studies which were claimed proprietary by the applicant are listed in Table 5.

Table 5: List of toxicological studies with the NF

Reference	Type of study	Test system	Dose
Unpublished Study No. AG200050 2020	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′-SL/plate (absence and presence of S9 mix)
Unpublished Study No. 200058 2020	Micronucleus study in bone marrow cells of mice (GLP, OECD TG 474 (2016a))	Slc:ICR(ICR) mice	500, 1,000 and 2,000 mg 3′-SL/kg bw
Unpublished Study No. CG220005 2022	*In vitro* micronucleus study in cultured mammalian cells (GLP, OECD TG 487 (2016b))	CHL/IU cells	500, 1,000 and 2,000 μg 3′-SL/mL (absence and presence of S9 mix)
Unpublished Study No. 100602RG 2021	90-day repeated dose oral toxicity study in rats (GLP, OECD TG 408 (2018))	SD rats	502, 1,003 and 2,007 mg 3′-SL/kg bw per day

bw: Body weight; SD: Sprague–Dawley; ICR: Institute of Cancer Research; CHL/IU: Chinese hamster lung cells.
Concentrations and doses are expressed as 3′-SL sodium salt (NF corrected for the purity).

The Panel also notes that in solution under acidic conditions, the NF will be hydrolysed to D-lactose and sialic acid (EFSA NDA Panel, 2020a, 2022a). The amount of these by-products produced during the digestion remains always lower than the intake estimated from human milk. Finally, the possible formation of 3′-sialyllactulose derived from 3′-SL by isomerisation of the terminal D-glucose moiety into D-fructose mainly under alkaline conditions during the production process (Zeng et al., 2018) is also considered not being of concern (EFSA NDA Panel, 2022a, 2023a).

[16] An Adequate Intake (AI) is the value set when there is not enough data to calculate an average requirement for a nutrient. An AI is the average level of intake of a nutrient, based on observations or experiments that is assumed to be adequate.

3.10.1. Genotoxicity

The *in vitro* assessment of the mutagenic potential of the NF was evaluated in a bacterial reverse mutation test (unpublished study report, 2020a). Two main tests, conducted as pre-incubation assays, were performed using *S*. Typhimurium strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2 uvrA, which were exposed to water or five different concentrations of the NF expressed as 3′-SL sodium salt (313, 625, 1,250, 2,500 or 5,000 μg/plate), either in the presence or absence of liver microsomal fractions (S9 mix). No reproducible or dose-related increases in revertant colony numbers over control counts were observed with any of the strains following exposure to the NF at any concentration (irrespective of the presence or absence of S9). Neither growth inhibition nor precipitation of the test substance was observed. Based on the results of the study, it was concluded that the NF is non-mutagenic at concentrations up to 5,000 μg 3′-SL/plate in the absence or presence of metabolic activation.

The potential ability of the NF to induce chromosome aberration in bone marrow of ICR (Institute of Cancer Research) mice was also assessed in an *in vivo* micronucleus (MN) study (unpublished study report, 2020b). After a dose-range finding study, the NF was administered twice (with a 24-h interval) by gavage at doses of 0 (water for injection), 500, 1,000 or 2,000 mg expressed as 3′-SL/kg bw to 5 male ICR mice/group. Animals were euthanised about 24 h after the second administration and femoral bone marrow smears were prepared and analysed. No statistically significant differences were noted in the frequency of micronucleated immature erythrocytes between the NF and negative control groups. No significant difference in the proportion of immature erythrocytes among total erythrocytes among study groups was observed. It was concluded that the NF does not have the potential for induction of chromosomal aberrations under the experimental condition applied. However, the Panel notes that the use of animals for this experiment was unnecessary as no evidence of the exposure of the bone marrow to the test substance was provided. Therefore, the applicant was requested to perform an additional *in vitro* MN test.

To investigate the MN-inducing potential of the NF in cultured mammalian cells, a study was conducted in Chinese hamster lung cells (CHL/IU) by a short-term treatment (6-h; with and without metabolic activation (S9 mix)) and long-term treatment (27-h) (unpublished study report, 2022). Concentrations of 500, 1,000 and 2,000 μg of the NF expressed as 3′-SL sodium salt/mL in physiological saline were used in the main test. No cytotoxicity (measurement of relative population doubling) was observed in any treatment method. No statistically significant increases in the number of binucleate cells containing micronuclei were noted after 6-h treatment in the presence of or absence of S9 mix or following 27-h treatment and remained within the background range. Therefore, the Panel concludes that the NF did not show any evidence of clastogenicity or aneugenicity in the absence and presence of metabolic activation up to the highest concentration of 2,000 μg 3′-SL/mL.

Taking into account the 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. Subchronic toxicity

The applicant provided a technical report of the 90-day study where groups of 10 Crl:CD(SD) rats/sex were given distilled water, 502, 1,003 and 2,007 mg of the NF expressed as 3′-SL sodium salt/kg bw per day by oral gavage (unpublished study report, 2021).

No deaths in the course of the study and no treatment-related changes in clinical signs, body weight, body weight gain or food consumption were observed in any rats. Also, functional observations, ophthalmological examination and oestrus cycle examination performed at the end or towards the end of the treatment period did not reveal any test-item related finding.

Some statistically significant changes (Appendix A) generally of small magnitude were noted in haematological parameters: decrease in PT (prothrombin time) and APTT (activated partial thromboplastin time) in high dose in males and an increase in percentage of basophils in the intermediate dose in males, and in clinical chemistry parameters with increase in γ-GPT (glutamyl transpeptidase) at intermediate dose in males and in glucose at the intermediate dose in females. At urinalysis variations in electrolytes were recorded: increased sodium concentration and sodium total excretion (low dose in females and in both sexes at the high dose), decreased potassium and chloride concentration (intermediate and high dose in males and females, respectively) and potassium and chloride total excretion (low and high dose in females). These changes observed were sporadic (increase in percentage of basophils, increase in γ-GPT, increase in glucose, increased sodium concentration and sodium total excretion, decreased potassium and chloride concentration) and/or

limited to only one sex (decrease in PT and APTT, increase in percentage of basophils, increase in γ-GPT, increase in glucose, decreased potassium and chloride total excretion), all of them were within historical control values and they are overall considered by the Panel as not toxicologically relevant.

There were no NF-related findings at gross necropsy in any animals. Some statistically significant changes were noted in both absolute and relative (to the bw) organ weights: decreased absolute brain weight at intermediate dose in males, decreased absolute and relative weight of pituitary and thyroid gland at low and intermediate doses in females, which the Panel considers as incidental and not treatment related.

At histopathology, changes were more frequently observed in rats receiving the high dose. Changes include mononuclear cell infiltration in the ventricular wall of the heart (1 M), focal fibrosis in the pancreas islets (7 M and 4 M in controls) or focal atrophy of acinar cells (2 M and 1 M in controls), unilateral scar in the kidney (1 M), unilateral cyst in the kidney medulla (1 M and 1 F), pseudocyst in the anterior lobe of the pituitary (1 M, 1 F and 1 F in controls), Rathke's pouch dilatation in the pituitary (4 F and 3 F in controls), focal mononuclear cell infiltration in the harderian gland (1 M), focal mononuclear cell infiltration in the prostate interstitium (2 M and 1 M in controls), focal fibrosis in the femur epiphysis (1 M). These findings were slight in severity, occurred in single animals (mononuclear cell infiltration in the heart, scar in the kidney, cyst in the kidney medulla, pseudocyst in the anterior lobe of the pituitary, focal mononuclear cell infiltration in the harderian gland, focal fibrosis in the femur epiphysis), in a single sex (focal fibrosis in the pancreas islets, focal atrophy of acinar cells, Rathke's pouch dilatation in the pituitary), in control animals (focal fibrosis in the pancreas islet, focal atrophy of acinar cells, pseudocyst in the anterior lobe of the pituitary, Rathke's pouch dilatation in the pituitary, focal mononuclear cell infiltration in the prostate interstitium) or were unilateral (scar in the kidney, cyst in the kidney medulla). Therefore, they were considered as incidental by the Panel.

The Panel considers that no adverse effects were observed in this study up to the highest tested dose of 2,007 mg 3′-SL sodium salt/kg bw per day.

3.10.3. Human data

No human intervention studies with the NF were provided by the applicant.

The applicant made reference to a publication where information from a randomised, double-blind, placebo-controlled clinical trial conducted with 3′-SL in adults with dyspeptic symptoms is reported. 60 dyspeptic adult patients positive to *Helicobacter pylori* consumed 0, 10 or 20 g 3′-SL/day divided into three administrations for 4 weeks (Parente et al., 2003). The authors concluded that 3′-SL was well tolerated at doses up to 20 g/day in adults (corresponding to about 290 mg/kg bw per day).

The Panel considers this information as supportive for the tolerability of 3′-SL in adults.

3.11. Allergenicity

The applicant did not find an allergenic potential of introduced proteins as a result of the genetic modification of the *E. coli* W (ATCC 9637) 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 3′-SL sodium salt, but it also contains sialic acid, D-lactose, D-glucose, 3′-sialyllactulose and 6′-SL sodium salts, and a small fraction of other related saccharides. The NF is produced by fermentation by a genetically modified strain (*E. coli* NEO3) of *E. coli* W (ATCC 9637).

The target population proposed by the applicant is the general population. The applicant intends to add the NF to a variety of foods, including IF and FOF, FSMP and FS. The applicant applies for the same uses and use levels already assessed for 3′-SL sodium salt produced by fermentation by a genetically modified strain of *E. coli* K-12 DH1, with the exception for the use as FS in individuals from 3 years of age for which the use level is proposed to be higher (from 0.5 to 1.0 g/day). Since the NF produced with the new process has similar composition and would be consumed as food ingredient at

the same extent as the already assessed 3′-SL in IF, FOF and other foods, no new estimates of the intake have been carried out. Similar considerations apply for the sodium intake. The newly proposed use as FS in individuals from 3 years of age of 1.0 g/day resulted in an estimated intake that is within the natural intake of 3′-SL in breastfed infants (EFSA NDA Panel, 2022a). The applicant stated that FS containing the NF are not intended to be used if other sources of 3′-SL are consumed on the same day. It is noted that additional sources for the oligosaccharides contained in the NF are cow milk and milk-derived products. However, the contribution from consumption of cow milk and milk-derived products is small (see Section 3.6.1).

The submitted toxicity studies did not raise safety concerns. No toxicologically relevant effects were observed in the subchronic toxicity study at up to the highest dose tested of 2,007 mg of the NF expressed as 3′-SL/kg bw per 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 breastfed 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.

5. Conclusions

The Panel concludes that the NF, which is composed of 3′-SL 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, LC–MS/MS and HPLC-CAD; (ii) production process; (iii) information on the genetically modified production strain; (iv) composition and stability of the NF; (v) toxicological (Table 4) and allergenicity studies.

6. Steps taken by EFSA

1) On 7 December 2021 EFSA received a letter from the European Commission with the request for a scientific opinion on the safety of 3′-sialyllactose (3′-SL) sodium salt as a novel food. Ref. Ares(2021)7548356.
2) On 7 December 2021, a valid application on the safety of 3′-sialyllactose (3′-SL) sodium salt as a novel food, which was submitted by Kyowa Hakko Bio Co., Ltd, was made available to EFSA by the European Commission through the Commission e-submission portal (NF 2021/2457) and the scientific evaluation procedure was initiated.
3) On 21 July 2022, EFSA requested the applicant to provide additional information to accompany the application and the scientific evaluation was suspended.
4) On 21 July 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 3 August 2023, the NDA Panel, having evaluated the data, adopted a scientific opinion on the safety of of 3′-sialyllactose (3′-SL) sodium salt as a novel food pursuant to Regulation (EU) 2015/2283.

References

Albrecht S, Lane JA, Marino K, Al Busadah KA, Carrington SD, Hickey RM and Rudd PM, 2014. A comparative study of free oligosaccharides in the milk of domestic animals. British Journal of Nutrition, 111, 1313–1328.

Aldredge DL, Geronimo MR, Hua S, Nwosu CC, Lebrilla CB and Barile D, 2013. Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. Glycobiology, 23, 664–676.

Archer CT, Kim JF, Jeong H, Park JH, Vickers CE, Lee SY and Nielsen LK, 2011. The genome sequence of *E. coli* W (ATCC 9637): comparative genome analysis and an improved genome-scale reconstruction of *E. coli*. BMC Genomics, 12, 20.

Bauer AP, Ludwig W and Schleifer KH, 2008. A novel DNA microarray design for accurate and straightforward identification of *Escherichia coli* safety and laboratory strains. Systematic and Applied Microbiology, 31, 50–61.

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2023. Scientific Opinion on the update of the list of qualified presumption of safety (QPS) recommended microorganisms intentionally added to food or feed as notified to EFSA. EFSA Journal 2023;21(1):7747, 23 pp. https://doi.org/10.2903/j.efsa.2023.7747

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

EFSA GMO Panel (EFSA Panel on Genetically Modified Organisms), 2010. Scientific opinion on the assessment of allergenicity of GM plants and microorganisms and derived food and feed. EFSA Journal 2010;8(7):1700, 168 pp. https://doi.org/10.2903/j.efsa.2010.1700

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2015a. Scientific opinion on the safety of 2′-O-fucosyllactose as a novel food ingredient pursuant to Regulation (EC) No 258/97. EFSA Journal 2015;13 (7):4184, 32 pp. https://doi.org/10.2903/j.efsa.2015.4184

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2015b. Scientific opinion on the safety of lacto-N-neotetraose as a novel food ingredient pursuant to Regulation (EC) No 258/97. EFSA Journal 2015;13(7):4183, 32 pp. https://doi.org/10.2903/j.efsa.2015.4183

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2015c. Statement on the safety of lacto-N-neotetraose and 2′-O-fucosyllactose as novel food ingredients in food supplements for children. EFSA Journal 2015;13(11):4299, 11 pp. https://doi.org/10.2903/j.efsa.2015.4299

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2019c. Scientific Opinion on the dietary reference values for sodium. EFSA Journal 2019;17(9):5778, 191 pp. https://doi.org/10.2903/j.efsa.2019.5778

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2016. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. EFSA Journal 2016;14(11):4594, 24 pp. https://doi.org/10.2903/j.efsa.2016.4594

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2017. Scientific opinion on the safety of synthetic N-acetyl-D-neuraminic acid as a novel food pursuant to Regulation (EC) No 258/97. EFSA Journal 2017;15(7):4918, 28 pp. https://doi.org/10.2903/j.efsa.2017.4918

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2019a. Scientific opinion on the safety of 2′-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283.EFSA Journal 2019;17(6):5717, 23 pp. https://doi.org/10.2903/j.efsa.2019.5717

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2019b. Scientific opinion on the safety of lacto-N-tetraose (LNT) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2019;17 (12):5907, 27 pp. https://doi.org/10.2903/j.efsa.2019.5907

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2020a. Scientific opinion on the safety of 3′-Sialyllactose (3′-SL) sodium salt as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2020;18(5):6098, 22 pp. https://doi.org/10.2903/j.efsa.2020.6098

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2020b. Scientific opinion on the safety of 6′-Sialyllactose (6′-SL) sodium salt as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2020;18(5):6097, 23 pp. https://doi.org/10.2903/j.efsa.2020.6097

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2020c. Scientific opinion on the safety of lacto-N-neotetraose (LNnT) produced by derivative strains of *E. coli* BL21 as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2020;18(11):6305, 11 pp. https://doi.org/10.2903/j.efsa.2020.6305

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2021. Scientific opinion on the safety of 3-Fucosyllactose (3-FL) as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2021;19 (6):6662, 25 pp. https://doi.org/10.2903/j.efsa.2021.6662

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2022a. Scientific Opinion on the safety of 3′-sialyllactose (3′-SL) sodium salt produced by derivative strains of *Escherichia coli* BL21 (DE3) as a Novel Food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2022;20(5):7331, 26 pp. https://doi.org/10.2903/j.efsa.2022.7331

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2022b. Scientific Opinion on the safety of 6′-sialyllactose (6′-SL) sodium salt produced by derivative strains of *Escherichia coli* BL21 (DE3) as anovel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2022;20(12):7645, 29 pp. https://doi.org/10.2903/j.efsa.2022.7645

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2022c. Scientific Opinion on the 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. EFSA Journal 2022;20(12):7647, 21 pp. https://doi.org/10.2903/j.efsa.2022.7647

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2022d. Scientific Opinion on the safety of the extension of use of 2′-fucosyllactose (2′-FL) and lacto-N-neotetraose (LNnT) as novel foods in food supplements for infants pursuant to Regulation (EU) 2015/2283. EFSA Journal 2022;20(5):7257, 9 pp. https://doi.org/10.2903/j.efsa.2022.7257

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2022e. Scientific Opinion on the safety of lacto-N-tetraose (LNT) produced by derivative strains of *Escherichia coli* BL21 (DE3) as a Novel Food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2022;20(5):7242, 24 pp. https://doi.org/10.2903/j.efsa.2022.7242

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2022f. Scientific opinion on the safety of the extension of use of 2′-fucosyllactose/difucosyllactose (2′-FL/DFL) mixture and lacto-N-tetraose (LNT) as novel foods in food supplements for infants pursuant to Regulation (EU) 2015/2283. EFSA Journal 2022;20(3):7140, 9 pp. https://doi.org/10.2903/j.efsa.2022.7140

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2022g. Scientific Opinion on the safety of 3-fucosyllactose (3-FL) produced by a derivative strain of *Escherichia coli* BL21 (DE3) as a Novel Food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2022;20(5):7329, 23 pp. https://doi.org/10.2903/j.efsa.2022.7329

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2023a. Scientific Opinion on the safety of 6′-sialyllactose (6′-SL) sodium salt produced by a derivative strain (*Escherichia coli* NEO6) of *E. coli* W (ATCC 9637) as a Novel Food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2023;21(6):8025, 23 pp. https://doi.org/10.2903/j.efsa.2023.8025

EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), 2023b. Scientific Opinion on the 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. EFSA Journal 2023;21(6):8026, 29 pp. https://doi.org/10.2903/j.efsa.2023.8026

EFSA Scientific Committee, 2012. Guidance on selected default values to be used by the EFSA Scientific Committee, Scientific Panels and Units in the absence of actual measured data. EFSA Journal 2012;10(3):2579, 32 pp. https://doi.org/10.2903/j.efsa.2012.2579

EFSA Scientific Committee, 2021. Guidance on technical requirements for regulated food and feed product applications to establish the presence of small particles including nanoparticles. EFSA Journal 2021;19(8):6769, 48 pp. https://doi.org/10.2903/j.efsa.2021.6769

Galuska CE, Rudloff S, Kuntz S, Borsch C, Reutzel M, Eckert G, Galuska SP and Kunz C, 2020. Metabolic fate and organ distribution of (13)C-3′-sialyllactose and (13)C-N-acetylneuraminic acid in wild-type mice – no evidence for direct incorporation into the brain. Journal of Functional Foods, 75, 104268. https://doi.org/10.1016/j.jff.2020.104268

NIH (National Institutes of Health), 2019. NIH guidelines for research involving recombinant or synthetic nucleic acid molecules. National Institutes of Health (NIH). Office of Science Policy, Bethesda, MD Available online: https://osp.od.nih.gov/biotechnology/nih-guidelines/ [Accessed: 15 April 2023].

OECD (Organisation for Economic Co-operation and Development), 1997. Test No. 471: Bacterial reverse mutation test. OECD guidelines for the testing of chemicals, Section 4: Health effects, 11.

OECD (Organisation for Economic Co-operation and Development), 1998. OECD Principles on Good Laboratory Practice, OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1. OECD Publishing, Paris. https://doi.org/10.1787/9789264078536-en

OECD (Organisation for Economic Co-operation and Development), 2016a. Test No. 474: mammalian erythrocyte micronucleus test, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/20745788

OECD (Organisation for Economic Co-operation and Development), 2016b. Test No. 487: In vitro mammalian cell micronucleus test, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264264861-en

OECD (Organisation for Economic Co-operation and Development), 2018. Test No. 408: Repeated dose 90-day oral toxicity study in rodents, OECD Guidelines for the Testing of Chemicals, Section 4. OECD Publishing, Paris. https://doi.org/10.1787/9789264070707-en

Parente F, Cucino C, Anderloni A, Grandinetti G and Bianchi Porro G, 2003. Treatment of *Helicobacter pylori* infection using a novel antiadhesion compound (3′sialyllactose sodium salt). A double blind, placebo-controlled clinical study. Helicobacter, 8, 252–256.

Röhrig CH, Choi SSH and Baldwin N, 2017. The nutritional role of free sialic acid, a human milk monosaccharide, and its application as a functional food ingredient. Critical Reviews in Food Science and Nutrition, 57, 1017–1038.

Unpublished study report, 2020a. Yoshimi Lab – Study No. AG200050. Bacterial reverse mutation test of 3′-SL sodium salt.

Unpublished study report, 2020b. Yoshimi Lab – Study No. 200058. Micronucleus Study of 3′-SL sodium salt in bone marrow cells of mice.

Unpublished study report, 2021. Higashimatsuyamai Lab – Study No. 100602RG. Ninety-day repeated oral dose toxicity study of 3′-SL sodium salt in rats.

Unpublished study report, 2022. Yoshimi Lab – Study No. CG220005. Micronucleus Study of 3′-SL sodium salt in cultured mammalian cells.

Urashima T, Taufik E, Fukuda K and Asakuma S, 2013. Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. Bioscience, Biotechnology, and Biochemistry, 77, 455–466.

US FDA, 2019. No GRN. 766 [3′-SL sodium salt; GeneChem Inc., Daejeon, Republic of Korea]. In: GRAS Notices. US Food and Drug Administration (US FDA), Center for Food Safety & Applied Nutrition (CFSAN), Office of Food Additive Safety, Silver Spring (MD). Available online: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/?set=GRASNotices&id=766&sort=GRN_No&order=DESC&startrow=1&type=basic&search=766

Zeng J, Hu Y, Jia T, Zhang R, Su T, Sun J, Gao J, Li G, Cao M and Song M, 2018. Chemoenzymatic synthesis of sialylated lactuloses and their inhibitory effects on Staphylococcus aureus. PLoS One, 13, e0199334. https://doi.org/10.1371/journal.pone.0199334

Abbreviations

1D	Mono-dimensional
2D	Two-dimensional
2′-FL	2′-Fucosyllactose
3-FL	3-Fucosyllactose
3′-SL	3′-Sialyllactose
6′-SL	6′-Sialyllactose
AAS	Atomic absorption spectroscopy
ADME	Absorption, Distribution, Metabolism and Excretion
AOAC	Association of Official Analytical Collaboration
APTT	Activated partial thromboplastin time
ATCC	American Type Culture Collection
BIOHAZ	EFSA Panel on Biological Hazards
bw	Body weight
CAS	Chemical Abstracts Service
CFU	Colony forming unit
CHL/IU	Chinese hamster lung cells
COSY	Correlation spectroscopy
Crl:CD(SD) rats	Charles River Laboratories: Caesarean-derived (Sprague Dawley) rats
DFL	Difucosyllactose
DM	Dry matter
DNA	Deoxyribonucleic acid
EC	European Commission
EFSA	European Food Safety Authority
Escherichia coli W	Waksman's *E. coli* strain
EU	Endotoxin unit
EU	European Union
F	Females
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
FOF	Follow-on formula
FS	Food supplements
FSMP	Food for special medical purposes
FSSC 22000	Food Safety System Certification 22000
Gal	Galactose
γ-GPT	Gamma glutamyl transpeptidase
Glc	Glucose
GLP	Good Laboratory Practices
GMO	EFSA Panel on Genetically Modified Organisms
GMP	Good Manufacturing Practices
GRAS	Generally Recognized As Safe
GRN	GRAS Notice
HACCP	Hazard Analysis Critical Control Points
HETCOR	Heteronuclear correlation
HiMO	Human-identical milk oligosaccharide
HMBC	Heteronuclear multiple-bond correlation
HMO	Human milk oligosaccharide
HPLC–CAD	High-performance liquid chromatography – charged aerosol detection
HPLC–PAD	High performance liquid chromatography – pulsed amperometric detection
ICP–MS	Inductively coupled plasma – mass spectrometry
ICP–OES	Inductively coupled plasma – optical emission spectroscopy

ICR	Institute of Cancer Research
IF	Infant formula
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
JP	Japanese Pharmacopoeia
LC	Liquid chromatography
LNnT	Lacto-N-neotetraose
LNT	Lacto-N-tetraose
LOD	Limit of detection
LOQ	Limit of quantification
M	Males
MN	Micronucleous
MS/MS	Tandem mass spectrometry
MW	Molecular weight
NANA, Neu5A	N-Acetyl-D-neuraminic acid, sialic acid
NBRC	National Biological Resource Center
ND	Not detected
NDA	EFSA Panel on Nutrition, Novel Foods and Food Allergens
NF	Novel food
NIH	National Institutes of Health
NMR	Nuclear magnetic resonance spectroscopy
OECD	Organisation for Economic Co-operation and Development
PT	Prothrombin time
qPCR	Quantitative polymerase chain reaction
QPS	Qualified presumption of safety
R_f	Retention factor
RH	Relative humidity
SD	Standard deviation
SD rats	Sprague Dawley rats
TG	Test guidelines
TOCSY	Total correlation spectroscopy
US	United States
US EPA	US Environmental Protection Agency
US FDA	US Food and Drug Administration
USP	US Pharmacopeia
w/w	Weight per weight

Appendix A – Summary of the 90-day oral toxicity study in rats

Study title	**GLP 90-day oral toxicity study on 3′-SL sodium salt in SD rats** (Unpublished study report, 2021)			
Key results				
Parameters	**Sex**	**Dose groups (expressed in mg 3′-SL sodium salt/kg bw per day)**		
		0 (control, G1); Mean ± SD	**502 (low dose, G2); Mean ± SD**	**1,003 (intermediate dose, G3); Mean ± SD**	**2,007 (high dose, G4); Mean ± SD**
Food consumption					
Study Day 14–15	M	26.2 ± 2.1	26.9 ± 2.8	26.9 ± 2.2	27.2 ± 1.8
	F	16.9 ± 2.9	17.5 ± 1.4	15.9 ± 2.0**	16.6 ± 1.9
Study Day 18–19	M	28.7 ± 1.5	29.1 ± 3.1	27.7 ± 2.1	26.7 ± 3.2
	F	18.1 ± 2.2	19.3 ± 1.5	17.9 ± 2.5*	19.0 ± 3.1
Haematology/Coagulation					
Basophils (%)	M	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1*	0.0 ± 0.0
	F	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PT (s)	M	10.6 ± 0.7	10.7 ± 1.1	10.5 ± 1.0	9.9 ± 0.2#
	F	9.6 ± 0.3	9.6 ± 0.3	9.6 ± 0.3	9.7 ± 0.2
APTT (s)	M	17.7 ± 1.3	17.0 ± 0.7	17.3 ± 1.0	16.2 ± 1.1**
	F	15.7 ± 1.8	14.8 ± 0.7	15.0 ± 0.5	15.1 ± 0.7
Clinical chemistry					
γ-GPT (IU/L)	M	2 ± 1	3 ± 1	3 ± 1*	2 ± 1
	F	3 ± 1	3 ± 1	3 ± 1	3 ± 1
Glucose (mg/dL)	M	178 ± 20	186 ± 21	179 ± 17	194 ± 23
	F	142 ± 23	157 ± 28	174 ± 15**	144 ± 21
Urinalysis					
Sodium (mmol/L)	M	70 ± 23	77 ± 32	89 ± 34	136 ± 67#
	F	90 ± 28	95 ± 26	92 ± 25	137 ± 41**
Sodium (mmol/24 h)	M	1.0 ± 0.4	1.2 ± 0.4	1.4 ± 0.4	2.3 ± 0.7**
	F	1.2 ± 0.2	0.9 ± 0.3	1.5 ± 0.2**	1.8 ± 0.3**
Potassium (mmol/L)	M	226.0 ± 59.2	195.6 ± 65.7	166.7 ± 26.0*	150.5 ± 46.9**
	F	259.3 ± 62.3	233.6 ± 61.6	188.7 ± 44.2	204.9 ± 68.9
Potassium (mmol/24 h)	M	3.1 ± 0.8	2.9 ± 0.7	2.6 ± 0.7	2.6 ± 0.4
	F	3.4 ± 0.6	2.3 ± 0.5**	3.2 ± 0.5	2.7 ± 0.4*

Chloride (mmol/L)	M	75 ± 27	62 ± 30	52 ± 18	49 ± 31
	F	119 ± 33	99 ± 29	84 ± 19*	83 ± 30*
Chloride (mmol/24 h)	M	1.1 ± 0.5	0.9 ± 0.4	0.8 ± 0.3	0.9 ± 0.4
	F	1.6 ± 0.3	1.0 ± 0.3**	1.4 ± 0.3	1.1 ± 0.3**
Organ weight values – absolute					
Brain (g)	M	2.26 ± 0.05	2.24 ± 0.09	2.15 ± 0.10*	2.19 ± 0.08
	F	1.94 ± 0.08	1.94 ± 0.09	1.94 ± 0.08	1.94 ± 0.06
Pituitary (mg)	M	19.1 ± 6.2	13.6 ± 1.6	17.7 ± 6.2	17.4 ± 5.0
	F	23.5 ± 5.5	16.3 ± 2.7**	18.3 ± 2.4**	24.2 ± 3.5
Thyroid (mg)	M	34.1 ± 9.2	36.3 ± 11.2	37.1 ± 9.1	32.9 ± 7.6
	F	33.4 ± 6.0	25.2 ± 4.2**	27.1 ± 4.6*	34.6 ± 6.1
Organ weight values – relative to bw					
Pituitary (mg/g bw)	M	0.0321 ± 0.0102	0.0226 ± 0.0034	0.0296 ± 0.0100	0.0290 ± 0.0088
	F	0.0796 ± 0.0139	0.0543 ± 0.0071**	0.0622 ± 0.0069**	0.0797 ± 0.0099
Thyroid (mg/g bw)	M	0.0570 ± 0.0133	0.0604 ± 0.0202	0.0622 ± 0.0156	0.0547 ± 0.0139
	F	0.1141 ± 0.0185	0.0843 ± 0.0140**	0.0929 ± 0.0187*	0.1145 ± 0.0220

SD: standard deviation; M: males; F: females.
*$p < 0.05$, **$p < 0.01$: significant difference in the parametric procedure; #$p < 0.05$; significant difference in the non-parametric procedure.