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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 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, Carmen Pelaez, Kristina Pentieva, Alfonso Siani, Frank Thies,
Sophia Tsabouri, Marco Vinceti, Francesco Cubadda, Thomas Frenzel, Marina Heinonen,
Rosangela Marchelli, Monika Neuhäuser-Berthold, Morten Poulsen, Miguel Prieto Maradona,
Josef Rudolf Schlatter, 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, but it also contains D-lactose, 3'-sialyllactulose, sialic acid, N-acetyl-D-glucosamine and a small fraction of other related oligosaccharides. The NF is produced by fermentation with two genetically modified strains of *Escherichia coli* BL21 (DE3), the production strain and the optional degradation strain. 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 infants and young children, food for special medical purposes and food supplements. The target population is the general population. The anticipated daily intake of 3'-SL 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'-SL from human milk in infants on a body weight basis. The intake of 3'-SL 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'-SL is also considered of no safety concern. Food supplements are not intended to be used if other foods with added 3'-SL or human milk are consumed on the same day. The Panel concludes that the NF is safe under the proposed conditions of use.

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

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Correspondence: nif@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, Carmen Pelaez, 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 13 May 2020, the company Chr. Hansen A/S submitted a request to the Commission in accordance with Article 10 of Regulation (EU) 2015/2283¹ 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.

In accordance with Article 10(3) of Regulation (EU) 2015/2283, the European Commission asks the European Food Safety Authority to provide a scientific opinion on the safety of 3'-SL sodium salt as a NF.

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

1.2. Additional information

3'-SL sodium salt is included in the Union list of authorised NFs (Commission Implementing Regulation (EU) 2017/2470²) when produced by fermentation with a genetically modified strain of *Escherichia coli* K-12 DH1. Since 2015, several scientific opinions have been adopted by the EFSA NDA Panel on the safety of human-identical milk oligosaccharides (HiMOs) as NFs pursuant to Regulation (EC) No 258/97 or Regulation (EU) 2015/2283: 2'-fucosyllactose (2'-FL) (EFSA NDA Panel, 2015a), lacto-N-neotetraose (LNnT) (EFSA NDA Panel, 2015b), LNnT and 2'-FL in food supplements (FS) for children (EFSA NDA Panel, 2015c), N-acetyl-D-neuraminic acid (NANA) (EFSA NDA Panel, 2017), 2'-FL/difucosyllactose (DFL) mixture (EFSA NDA Panel, 2019a), lacto-N-tetraose (LNT) produced with a derivative strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019b), 3'-sialyllactose (3'-SL) sodium salt produced with a derivative strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2020a), 6'-sialyllactose (6'-SL) sodium salt (EFSA NDA Panel, 2020b), LNnT produced with derivative strains of *E. coli* BL21 (DE3) (EFSA NDA Panel, 2020c), 3-fucosyllactose (3-FL) (EFSA NDA Panel, 2021), 2'-FL/DFL mixture and LNT in FS for infants (EFSA NDA Panel, 2022a), 2'-FL and LNnT in FS for infants (EFSA NDA Panel, 2022b) and LNT produced with derivative strains of *E. coli* BL21 (DE3) (EFSA NDA Panel, 2022c).

2. Data and methodologies

2.1. Data

The safety assessment of this NF is based on data supplied in the application and information submitted by the applicant following an EFSA request for supplementary information.

During the assessment, the Panel identified additional data which were not included in the application.

Administrative and scientific requirements for NF applications referred to in Article 10 of Regulation (EU) 2015/2283 are listed in the Commission Implementing Regulation (EU) 2017/2469³.

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 (both in favour and not in favour) that are pertinent to the safety of the NF.

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

¹ Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001. OJ L 327, 11.12.2015, p. 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, p. 72–201.

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, p. 64–71

strain and the genetically modified optional degradation strain; (iv) method validation reports for the determination of the carbohydrate content in the NF; (v) clinical study report on suitability and tolerability of infant formula (IF) containing a HIMO mixture.

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 the Commission Implementing Regulation (EU) 2017/2469. The legal provisions for the assessment of food intended for infants and young children and food for special medical purposes (FSMP) are laid down in Regulation (EU) No 609/2013⁴ and, respectively, in Commission Delegated Regulation (EU) 2016/128⁵ (FSMP), and in Commission Delegated Regulation (EU) 2016/127⁶ (as regards the specific compositional and information requirements for 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. Furthermore, this assessment also is not an assessment on whether the NF is suitable as stipulated by Regulation (EU) No 609/2013.

3. Assessment

3.1. Introduction

The NF's primary constituent is the sodium salt of 3'-SL, henceforth named '3'-SL sodium salt' ($\geq 88.0\%$ 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 acknowledged as human milk oligosaccharides (HMOs). 3'-SL is a sialylated (acidic) trisaccharide composed of α -D-glucose, α -D-galactose and NANA (hereinafter also referred to as 'sialic acid'). The Panel notes that although 3'-SL sodium salt is the major component of the NF, related substances, namely α -D-lactose, 3'-sialyllactulose, sialic acid, N-acetyl-D-glucosamine and a small fraction of other related saccharides, are also present. The NF is produced by fermentation with two derivative strains of *E. coli* BL21 (DE3), the production strain and the optional degradation strain, and is isolated as a purified ingredient in the sodium salt form.

The NF is proposed to be used in food for infants and young children (including IF, FOF, processed cereal-based food and baby food as defined in Regulation (EU) No 609/2013), FSMP as defined in Regulation (EU) No 609/2013 and FS as defined in Directive 2002/46/EC⁷. The target population is the general population.

Sodium salts of 3'-SL (EFSA NDA Panel, 2020a) and 6'-SL (EFSA NDA Panel, 2020b), produced with derivative strains of *E. coli* K-12 DH1, have been previously assessed by EFSA as NFs, with positive outcomes. In addition, 2'-FL and LNnT (EFSA NDA Panel, 2020c), produced with derivative strains of the same host strain *E. coli* BL21 (DE3), have been authorised as NFs in the European Union (Commission Implementing Regulation 2017/2470), and LNT produced with derivative strains of *E. coli* BL21 (DE3) has recently been assessed by EFSA with a positive outcome (EFSA NDA Panel, 2022c).

According to Article 3(2)(a) of Regulation (EU) 2015/2283, the NF falls under the following categories:

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, p. 35–56.

⁵ 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.

⁶ 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.

⁷ Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements. OJ L 183, 12.7.2002, p. 51–57.

- 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 ($\geq 88.0\%$ w/w DM), but it also contains D-lactose ($\leq 5.0\%$ w/w DM), 3'-sialyllactulose ($\leq 5.0\%$ w/w DM), sialic acid ($\leq 1.5\%$ w/w DM), N-acetyl-D-glucosamine ($\leq 1.0\%$ w/w DM) and a small fraction of other related saccharides (sum of other carbohydrates $\leq 5.0\%$ w/w DM). It is produced by fermentation with two genetically modified strains of *E. coli* BL21 (DE3), the production strain and the optional degradation strain. 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) rather than α -(2-3).

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

Chemical substance	
Chemical (IUPAC) name	N-acetyl- α -D-neuraminyl-(2→3)- β -D-galactopyranosyl-(1→4)-D-glucopyranose, sodium salt
Common name	3'-Sialyllactose, sodium salt
Abbreviations	3'-SL, sodium salt
Alternative chemical names	<ul style="list-style-type: none"> • 3'-SL sodium salt • 3'-N-acetylneuraminyl-D-lactose sodium salt • α-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 weight	655.53 Da

The molecular structure of 3'-SL has been determined by high-performance liquid chromatography – electrospray ionisation – tandem mass spectrometry (HPLC-ESI-MS/MS), based on its collision-induced decay (CID) fragmentation pattern and multiple reaction monitoring (MRM) analysis, by comparison with high purity in-house and commercially available standards.

The identity of 3'-SL was also confirmed by high-performance anion-exchange chromatography – pulsed amperometric detection (HPAEC-PAD) by comparison with a high purity in-house standard.

The structure of 3'-SL has been confirmed by mono-dimensional (1D) nuclear magnetic resonance (NMR) spectroscopy including ¹H, ¹³C and ¹³C-DEPT-90 (distortionless enhancement by polarisation transfer) spectra, and two-dimensional (2D) NMR spectroscopy, including ¹H-¹H-COSY (correlated spectroscopy), ¹H-¹³C-HSQC (heteronuclear single quantum correlation), ¹H-¹³C-HMBC (heteronuclear multiple-bond correlation) and ¹H-¹³C HSQC-TOCSY (heteronuclear single quantum correlation total correlation spectroscopy) spectra. In particular, some correlations have been identified from COSY spectra. All correlations for carbons involved in glycosidic linkages were evidenced in the HSQC spectrum and the interglycosidic correlations showing the inter-residue interactions were confirmed by the HMBC spectrum. Taken together, NMR data confirm that the glycosidic linkage between Neu5Ac C-2 and the adjacent D-galactose (Gal) H-3' is α -(2-3). An inter-residual correlation was also observed between Gal H-1' and D-glucose (Glc) C-4 in the HMBC spectrum. This, together with the coupling constants of the Gal H-1' and the Glc H-1 signals, indicates that: (i) the link between the Gal and the Glc units is β -(1-4); (ii) the pyranose configuration is β for the Gal unit; and (iii) the terminal D-glucose in water solution is in equilibrium between the α - and the β -anomeric forms.

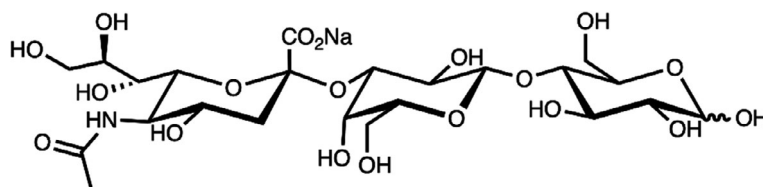


Figure 1: Chemical structure of 3'-SL sodium salt (EFSA NDA Panel, 2020a)

The 3'-SL produced by the microbial fermentation described has been shown to be chemically and structurally identical to a commercially available 3'-SL derived from human milk by 1D and 2D NMR spectroscopy, and the Panel considers it as being a HiMO.

3.3. Production process

According to the information provided by the applicant, the NF is produced in line with Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Points (HACCP) principles, in a facility that is ISO:9001 and FSSC 22000 certified.

The NF is produced by a two-step fed-batch fermentation process using two genetically modified strains derived from the host strain *E. coli* BL21 (DE3). These strains are the 'production strain' *E. coli* BL21 (DE3) PS-3'-SL-JBT and the optional 'degradation strain' *E. coli* BL21 (DE3) DS-3'-SL-JBT. The production strain has been modified to effectively synthesise 3'-SL, while the optional degradation strain is equipped with enzymes to degrade intermediate carbohydrate by-products and remaining substrates in order to facilitate the production process. Glycerol, glucose and/or sucrose can be used as carbon sources for the cultivation of both strains and lactose is utilised as a substrate for the production of 3'-SL by the production strain. The process is carried out without inhibitors, inducers or antibiotics and no solvents are used except water. The duration of the fermentation step is set to optimise the concentration of 3'-SL. At the end of the fermentation process, the bacterial biomass is removed from the final product by centrifugation and ultrafiltration. The isolation, purification and concentration of the product involve several filtration, ion removal and decolourisation steps. All chemicals used in the process are of food-grade quality. Other processing aids, such as ion exchange resins, activated carbon and filtration membranes, are also in conformance with the manufacture of food. The concentrated purified 3'-SL sodium salt is spray-dried to obtain a powder form.

The production and the optional degradation strains *E. coli* BL21 (DE3) PS-3'-SL-JBT and *E. coli* BL21 (DE3) DS-3'-SL-JBT, respectively, are genetically modified derivatives of the host strain *E. coli* BL21 (DE3) ($F^- ompT hsdS_B (r_B^- m_B^-) gal dcm$ (DE3)). The *E. coli* BL21 (DE3) strain was developed through T7 RNA polymerase-based gene expression by introducing a lambda prophage containing a T7 RNA polymerase under the control of *lacUVA* promoter and it is typically used in laboratories worldwide. *E. coli* BL21 (DE3) is considered to be non-pathogenic and unlikely to survive in host tissues or to cause disease (Chart et al., 2000). The genome sequence of *E. coli* BL21 (DE3) showed the absence of genes encoding invasion factors, adhesion molecules and enterotoxins associated with virulence (Jeong et al., 2009). Both the production and the optional degradation strains have been deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ). A detailed description of the genetic modification steps applied to obtain both the production and the optional degradation strains has been provided by the applicant. No residual DNA from the production and the optional degradation strains was detected in the NF using quantitative polymerase chain reaction (qPCR) amplification of (a) four antimicrobial resistance genes introduced during the genetic modification of the production strain and (b) a specific DNA sequence for the optional degradation strain. The absence of both DNA and viable cells from the production and the optional degradation strains has been demonstrated in accordance with the EFSA Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA FEEDAP Panel, 2018).

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

3.4. Compositional data

Batch-to-batch analyses showed that the NF consists of 3'-SL sodium salt as primary ingredient (92.4% w/w DM⁸ as sodium salt). The remainder is a mixture of substances^{8,9} such as D-lactose (0.3% w/w DM), 3'-sialyllactulose (< 1.13% w/w DM), sialic acid (0.2% w/w DM) and N-acetyl-D-glucosamine (< 0.14% w/w DM). In addition, the NF contains other carbohydrates individually present at low concentration (sum of other carbohydrates, 1.4% w/w DM^{8,9}). 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. Sialic acid is an endogenous human and ubiquitous nutritional monosaccharide (EFSA NDA Panel, 2017; Röhrig et al., 2017), while 3'-sialyllactulose is 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). N-acetyl-D-glucosamine is also present in human milk as a building block for oligosaccharides (Garrido et al., 2012).

With regard to the physico-chemical properties, the NF can be described as a white- to ivory-coloured spray-dried powder. It is readily soluble in aqueous solutions (min. 500 g/L in water at ambient temperature).

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 10 batches of the NF (Table 2). Information was provided on the accreditation of the laboratories that conducted the analyses presented in the application.

⁸ Average content in 10 batches of the NF.

⁹ For those batches of the NF where the levels of any carbohydrate by-product were below the respective limit of quantification (LOQ), the concentration of the corresponding compound has been considered to be equal to the respective LOQ value for the purpose of calculating its average content in the 10 batches of the NF and the sum of other carbohydrates.

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

Parameters	Batches										Method of analysis
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10	
Composition											
3'-SL sodium salt (% w/w DM)	93.9	94.1	93.8	91.4	92.1	91.3	88.1	96.8	92.1	90.1	HPAEC-PAD (validated internal method) ¹
D-Lactose (% w/w DM)	0.3	0.3	0.4	0.4	0.3	< LOQ	0.5	< LOQ	< LOQ	< LOQ	
Sialic acid (% w/w DM)	0.2	0.3	0.3	0.4	0.2	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
N-acetyl-D-glucosamine (% w/w DM)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
3'-Sialyllactulose (% w/w DM)	–	–	–	–	–	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	Calculation ²
Sum of other carbohydrates (% w/w DM)	1.8	1.1	2.0	4.8	1.2	0.1	3.1	0.0	0.3	0.0	
Protein (%)	0.001	0.002	0.001	0.002	0.002	0.001	0.002	0.002	0.002	0.002	Nanoquant (modified Bradford)
Ash (%)	3.7	4.1	3.4	2.9	6.1	7.1	6.9	3.3	6.1	8.4	ASU L 06.00-4
Water (%)	8.3	8.8	8.5	8.7	6.0	6.3	6.9	6.3	6.0	6.4	Karl Fischer titration
Sodium (%)	3.0	3.1	3.0	3.2	2.8	3.1	3.1	3.4	2.8	3.0	ICP-MS (ASU L 00.00-13)
Contaminants											
Arsenic ³ (mg/kg)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	ASU L 00.00-135: 2011-01 – ICP-MS
Cadmium ³ (mg/kg)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
Lead ³ (mg/kg)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	0.011	
Mercury ³ (mg/kg)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
Aflatoxin M1 (µg/kg)	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	DIN EN ISO 14501: 2008-01 – IAC-HPLC-FD
Microbial parameters											
Standard plate count (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	ISO 4833-2
Yeast and mould (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	ISO 21527-2: 2008-07
<i>Enterobacteriaceae</i> (CFU/g)	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	< 10	ISO 21528-2: 2019-05
<i>Salmonella</i> (in 25 g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	DIN EN ISO 6579-1: 2017-07
<i>Cronobacter</i> spp. (in 10 g)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ISO/TS 22964: 2017-04
<i>Listeria monocytogenes</i> (in 25 g)	ND	–	–	ND	ND	–	–	–	–	–	DIN EN ISO 11290-1: 2017-09
<i>Bacillus cereus</i> (in 5 g)	ND	–	–	ND	ND	–	–	–	–	–	ASU L 00.00-108: 2007-04
Endotoxins (EU/mg)	0.018	0.010	0.011	0.026	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	0.289	Ph. Eur. 2.6.14

–: Not reported; 3'-SL: 3'-Sialyllactose; ASU: Official collection of analysis methods according to § 64 of the German Food and Feed Code (LFGB); CFU: Colony forming unit; DIN: German Institute for Standardisation e. V.; DM: Dry matter; EN: European norm; EU: Endotoxin unit; HPAEC-PAD: High-performance anion-exchange chromatography – pulsed amperometric detection; IAC-HPLC-

FD: Immunoaffinity chromatography – high-performance liquid chromatography – fluorescence detector; ICP-MS: Inductively coupled plasma – mass spectrometry; ISO: International Organisation for Standardisation; LOQ: Limit of quantification; ND: Not detected; Ph. Eur.: European Pharmacopeia; TS: Technical specification.

1: LOQs: D-Lactose = 0.14% w/w DM; Sialic acid = 0.14% w/w DM; N-acetyl-D-glucosamine = 0.14% w/w DM; 3'-Sialyllactulose = 1.13% w/w DM.

2: Sum of other carbohydrates = 100 (% w/w DM) – 3'-SL sodium salt (% w/w DM) – Quantified carbohydrates (% w/w DM) – Ash (% w/w DM). 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 the sum of other carbohydrates in the corresponding batch.

3: LOQs: Arsenic = 0.05 mg/kg; Cadmium = 0.010 mg/kg; Lead = 0.010 mg/kg; Mercury = 0.005 mg/kg.

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

3.4.1. Stability

Stability of the NF

The applicant performed stability tests on one batch of a HiMO mixture containing 2'-FL (47.7% w/w DM), 3-FL (15.1% w/w DM), LNT (24.7% w/w DM), 3'-SL sodium salt (4.3% w/w DM), 6'-SL sodium salt (5.6% w/w DM) and other carbohydrates (5.7% w/w DM). The applicant stated that 3'-SL sodium salt included in the HiMO mixture was manufactured according to the production process described in Section 3.3. The tests were carried out at normal (25°C and 60% relative humidity (RH)) and accelerated (40°C and 75% RH) storage conditions for a period of 104 and 26 weeks, respectively. The samples were analysed for 3'-SL (HPAEC-PAD) and moisture (Karl-Fischer titration) content. Upon EFSA's request for additional information, the applicant provided stability data up to 156-week storage under normal conditions, also reporting the concentration of the individual carbohydrates present in the HiMO mixture throughout the storage period.

The content of 3'-SL (expressed on a DM basis) remained relatively stable over the 156-week period under normal storage conditions (average content of 5.4% w/w DM), with an increase in the moisture content from 5.7% to 10.9%, which exceeds the specifications ($\leq 9.0\%$). Over the 26-week storage under accelerated conditions, the content of 3'-SL (expressed on a DM basis) remained relatively unchanged (average content of 5.5% w/w DM), and an increase from 5.7% to 9.9%, again above the specifications, was observed for the moisture content. Under both normal and accelerated conditions, the total concentration and composition of the HiMO mixture (expressed on a DM basis) remained relatively constant.

The applicant was also requested to provide microbiological analysis, in light of the increase in the moisture content throughout the storage period. Thus, three batches of the NF and three batches of the above-mentioned HiMO mixture stored under warehouse conditions for 25 months were analysed for total viable counts, yeasts and moulds. Microbial levels were below the respective limits of detection and the moisture content was within the specifications (average content of $8.7 \pm 1.2\%$ in the NF and $6.6 \pm 1.2\%$ in the HiMO mixture).

The applicant also referred to the stability studies included in the GRAS (Generally Recognised As Safe) notifications GRN (GRAS Notice) 766 and GRN 880 (US FDA, 2019, 2020) on 3'-SL sodium salt produced by enzymatic synthesis or fermentation with a derivative strain of *E. coli* K-12 DH1, respectively, as well as to the stability of the authorised NF for at least 24 months when stored at room temperature (EFSA NDA Panel, 2020a). The applicant proposed a 1-year shelf-life from the date of production of the NF, when stored under ambient conditions.

The Panel considers that the data provided sufficient information with respect to the stability of the NF for 1 year.

Stability of the NF under the intended conditions of use

A stability study was conducted with two batches of powdered IF and three batches of a ready-to-use liquid IF using the above-mentioned HiMO mixture, which contains the NF. The concentration of the individual HiMOs (HPAEC-PAD) and pH levels were determined immediately after production and after 3- and 6-month storage at ambient conditions. The content of 3'-SL sodium salt remained relatively constant over the storage period and its stability in IF was demonstrated up to 6 months at ambient conditions.

No stability data for 3'-SL sodium salt in other food matrices were provided. The NDA Panel concluded in its previous assessment of this HiMO that 'the available data provide sufficient information with respect to the stability of the NF in the food matrices at neutral pH, when stored at room temperature under proper storage conditions' (EFSA NDA Panel, 2020a).

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

3.5. Specifications

The specifications of the NF are indicated in Table 3.

Table 3: Specifications of the NF

Parameter	Specification
Description: 3'-Sialyllactose (3'-SL) sodium salt is a white- to ivory-coloured powder produced by microbial fermentation and further isolated, purified and concentrated.	
Source: Two genetically modified strains of <i>Escherichia coli</i> BL21 (DE3).	
Composition	
3'-SL sodium salt (% w/w DM)	≥ 88.0
3'-Sialyllactulose (% w/w DM)	≤ 5.0
D-Lactose (% w/w DM)	≤ 5.0
Sialic acid (% w/w DM)	≤ 1.5
N-acetyl-D-glucosamine (% w/w DM)	≤ 1.0
Sum of other carbohydrates ¹ (% w/w DM)	≤ 5.0
Water (%)	≤ 9.0
Protein (%)	≤ 0.01
Ash (%)	≤ 8.5
Sodium (%)	≤ 4.2
Contaminants	
Arsenic (mg/kg)	≤ 0.2
Aflatoxin M1 (µg/kg)	≤ 0.025
Microbiological criteria	
Standard plate count (CFU/g)	≤ 1,000
Yeast and mould (CFU/g)	≤ 100
<i>Enterobacteriaceae</i> (CFU/g)	≤ 10
<i>Salmonella</i> (in 25 g)	ND
<i>Cronobacter (Enterobacter) sakazakii</i> (in 10 g)	ND
Endotoxins (EU/mg)	≤ 10

3'-SL: 3'-Sialyllactose; CFU: Colony forming unit; DM: Dry matter; EU: Endotoxin unit; ND: Not detected.

1: Sum of other carbohydrates = 100 (% w/w DM) – 3'-SL sodium salt (% w/w DM) – Quantified carbohydrates (% w/w DM) – Ash (% w/w DM). 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 the sum of other carbohydrates.

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 produced by fermentation with a genetically modified strain of *E. coli* K-12 DH1 has been authorised as an NF in the European Union (Commission Implementing Regulation 2021/96¹⁰) to be added to IF and FOF, to a variety of foods as well as to FS.

3'-SL has been also 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).

¹⁰ 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.

3.6.2. Intake of 3'-SL from human milk

As reported in previous EFSA opinions (EFSA NDA Panel, 2019b, 2020a,b, 2021), 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; Urashima et al., 2018). Concentration and composition of HMOs vary across mothers and over the course of lactation. 3'-SL belongs to the subfraction of 'acidic' HMOs, which is characterised by the presence of sialic acids, and the whole subfraction accounts for 1.5–3.3 g/L (Thurl et al., 2010; Rijniere et al., 2011; Bode, 2012).

There are two naturally occurring sialyllactoses that are constitutional isomers with a minimal structural difference. The oligosaccharide backbone can be sialylated by α -(2-3) or α -(2-6) linkages, resulting in 3'-SL or 6'-SL, respectively. The two forms have been shown to have similar functions and biological roles (Tarr et al., 2015). Several publications on HMOs and 3'-SL in human milk have been provided by the applicant.

The highest concentration of 3'-SL in human milk is reported in colostrum, and as for other HMOs, the concentration is genetically determined and also depends on the stage of lactation (Coppa et al., 1999, 2011; Asakuma et al., 2007; Thurl et al., 2010, 2017; Spevacek et al., 2015; Austin et al., 2016; Kunz et al., 2017; McGuire et al., 2017). Thurl et al. (2017) summarised the findings from 21 studies and reported that the mean concentration of 3'-SL in milk from mothers who delivered at term ranged from 0.14 to 0.24 g/L (average 0.19 g/L). It was noted that the range of 3'-SL concentrations was slightly wider (from 0.21 to 0.36 g/L, average 0.29 g/L) in mothers who delivered preterm (Thurl et al., 2017). Other publications reported maximum concentrations in European human milks up to 0.51 (average 0.10–0.28 g/L; Austin et al., 2019) or 0.60 g 3'-SL/L (average 0.13–0.25 g/L; Samuel et al., 2019). In a recent review (Soyyilmaz et al., 2021), a mean of mean concentrations of 0.19 g/L has been reported for mature milk, with a maximum mean of 0.70 g/L.

In consideration of the large and recent data set used in this review (Soyyilmaz et al., 2021), the Panel decided to use the values corresponding to the mean of means (0.19 g/L) and the maximum mean (0.70 g/L) as representative of the average natural concentrations found in mature human milk.

Based on these reported concentrations of 3'-SL 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'-SL 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'-SL from average (800 mL) and high (1,200 mL) human milk intakes for infants of 6.7 kg bw, based on mean and high concentrations of 3'-SL of 0.19 g/L and 0.70 g/L, respectively, in mature human milk (Soyyilmaz et al., 2021)

	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'-SL	23	84	34	125

bw: body weight.

The Panel noted that although the main component of the NF is 3'-SL sodium salt, other fractions such as D-lactose, sialic acid, 3'-sialyllactulose and N-acetyl-D-glucosamine are present in different amounts (see Section 3.4).

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 IF and FOF, processed cereal-based food and baby food for infants and young children and milk-based drinks and similar products intended for young children. These food products, defined using the FoodEx2¹¹ hierarchy, and the maximum use levels are reported in Table 5.

The applicant also intends to market the NF in FS, at the maximum daily intake of 0.70 g 3'-SL sodium salt for individuals above 3 years of age or at a maximum daily intake of 0.23 g 3'-SL sodium salt when intended for infants (up to 11 months) or young children (12–35 months).

For the category FSMP, the applicant proposed the use in accordance with the particular nutritional requirements of the persons for whom the products are intended according to Regulation (EU) No 609/2013.

According to the applicant, FS are not intended to be used 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	Max use level (mg NF/100 g)
A03PZ	4	Infant formulae, powder	184 ^(a)
A03QE	4	Infant formulae, liquid	23
A03QK	4	Follow-on formulae, powder	224 ^(a)
A03QQ	4	Follow-on formulae, liquid	28
A03QZ	3	Cereals with an added high protein food which have to be reconstituted with water or other protein-free liquid	112 ^(a)
A03QY	3	Simple cereals which have to be reconstituted with milk or other appropriate nutritious liquids	196 ^(a)
A0BZF	3	Cereals with an added high protein food reconstituted	28
A0BZE	3	Simple cereals for infants and children, reconstituted	28
A03RA	3	Biscuits, rusks and cookies for children	28
A03RB	3	Pasta for children (dry, to be cooked)	28
A03RH	3	Ready-to-eat dairy-based meal for children	28
A03RP	3	Special food for children's growth	28
A03RN	3	Fruit and vegetable juices and nectars specific for infants and young children	28

(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'-SL sodium salt 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 (0.23 g/L in IF), the high intake of the NF from IF alone is estimated to be 60 mg/kg bw per day.

The Panel notes that the anticipated daily intake of the NF from the consumption of IF does not exceed the estimated high daily intake of 3'-SL of 125 mg/kg bw in breastfed infants (Table 4).

Anticipated intake of 3'-SL sodium salt from the proposed uses and use levels of the NF

EFSA performed an assessment of the anticipated daily intake of the NF based on the applicant's proposed uses and maximum proposed use levels (Table 5), using individual data from the EFSA Comprehensive European Food Consumption Database (EFSA, 2011). The lowest and highest mean

¹¹ FoodEx2 is an EFSA standardised food classification and description system <https://www.efsa.europa.eu/en/data/data-standardisation>

and 95th percentile anticipated daily intakes of the NF (on a mg/kg 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 supporting information).

Table 6: Intake estimate resulting from the use of 3'-SL sodium salt 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	5.7	24.8	20.3	50.9
Young children ^(c)	1 to < 3	0.4	9.3	3.3	22.5
Other children	3 to < 10	0.0	0.4	0.0	3.1
Adolescents	10 to < 18	0.0	0.1	0.0	0.8
Adults ^(d)	≥ 18	0.0	0.0	0.0	0.2

(a): Intakes were assessed for all EU dietary surveys available in the food comprehensive database on 18 February 2022. The lowest and the highest averages observed among all EU surveys are reported in these columns.

(b): Intakes were assessed for all EU dietary surveys available in the food comprehensive database on 18 February 2022. 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'-SL sodium salt in the NF accounts for about 92.4%; therefore, the figures that are calculated considering a 100% purity slightly overestimate the actual intake. The Panel notes that the anticipated daily intake of 3'-SL sodium salt from the proposed uses and use levels does not exceed the estimated high daily intake of 125 mg/kg bw of 3'-SL in breastfed infants (Table 4).

3.7.4. Anticipated intake of the NF from food supplements

The applicant has proposed a maximum daily intake of the NF of 0.70 g/day in FS for individuals above 3 years of age or a maximum use level of 0.23 g/day when intended for infants (up to 11 months) and young children (12–35 months).

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 (mg/day)	Intake (mg/kg bw per day) ^(b)
Infants	< 1	5.0	230	46
Young children ^(c)	1 to < 3	12.0	230	19
Other children	3 to < 10	23.1	700	30
Young adolescents	10 to < 14	43.4	700	16
Old adolescents	14 to < 18	61.3	700	11
Adults	≥ 18	70.0	700	10

(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' is calculated by considering the use levels in 'mg/day' 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 daily intake of the NF from its use in FS (i.e. from 230 to 700 mg/day) for any population category (Table 7) does not exceed the estimated high daily intake of 3'-SL of 125 mg/kg bw in breastfed infants (Table 4).

According to the applicant, FS containing the NF are not intended to be used if other foods with added 3'-SL are also consumed on the same day. The Panel similarly notes that infants and young children should not consume human milk and the FS on the same day.

3.7.5. Combined intake from the NF and other sources

The Panel notes that 3'-SL sodium salt is already authorised for use in food categories other than those proposed for the NF under assessment (e.g. use in beverages, flavoured and unflavoured fermented milk-based products, cereal bars)².

The combined daily intake of 3'-SL sodium salt 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 supporting information). Therefore, the combined intake of 3'-SL sodium salt from already authorised uses and the currently proposed uses is higher (highest P95th intake of 77.3 mg/kg bw in young children, Table 8) than the estimated intake based on only the currently proposed uses and use levels (highest P95th intake of 50.9 mg/kg bw in infants, Table 6).

Table 8: Intake estimate resulting from the combined uses of 3'-SL sodium salt 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	8.8	41.0	22.0	75.9
Young children ^(c)	1 to < 3	5.6	22.8	13.3	77.3
Other children	3 to < 10	2.3	8.2	5.1	14.1
Adolescents	10 to < 18	0.5	3.2	2.0	7.4
Adults ^(d)	≥ 18	1.3	1.7	2.9	4.1

(a): Intakes were assessed for all EU dietary surveys available in the food comprehensive database on 18 February 2022. The lowest and the highest averages observed among all EU surveys are reported in these columns.

(b): Intakes were assessed for all EU dietary surveys available in the food comprehensive database on 18 February 2022. 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 in infants and young children from the combined exposure (i.e. 75.9 and 77.3 mg/kg bw, respectively; Table 8) from the maximum authorised and proposed uses, is higher than the estimated daily intake from the authorised uses alone (i.e. 71.3 and 70.4 mg/kg bw, respectively; EFSA NDA Panel, 2020), and below the high estimate for 3'-SL daily intake from human milk (i.e. 125 mg/kg bw; Table 4).

Additional sources for the oligosaccharides contained in the NF will be human milk, cow milk, fermented milk-based products and selected cheeses retaining milk sugar (e.g. curd cheese). However, in comparison to the natural intake of 3'-SL from human milk (Table 4) and the intake from the suggested uses and use levels of the NF, the contribution from consumption of cow milk and milk-derived products is small.

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

No ADME data have been provided for the NF.

As reported in previous EFSA opinions (EFSA NDA Panel, 2019a,b,c, 2020a,b, 2021), HMOs, including 3'-SL, are considered 'non-digestible oligosaccharides' (EFSA NDA Panel, 2014) since they do not undergo any significant digestion in the upper gastrointestinal tract (Brand-Miller et al., 1995, 1998; Engfer et al., 2000; Gnoth et al., 2000; Chaturvedi et al., 2001; Rudloff and Kunz, 2012).

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

Based on information available on HMOs, the Panel considers that limited digestion of the NF occurs in the gastrointestinal tract and that only small amounts are expected to be absorbed. Moreover, there are no indications that the absorption of 3'-SL, which is the main constituent of the NF, or other structurally related mono- and oligosaccharides (e.g. D-lactose), differs from that of similar components in human milk (EFSA NDA Panel, 2020a).

3.9. Nutritional information

The NF is mainly composed by the non-digestible oligosaccharide 3'-SL.

The Panel notes that the NF, being a sodium salt, may contribute to the daily sodium intake. In its opinion on DRVs for sodium, the NDA Panel has provided advice on levels of sodium intake that are considered safe and adequate¹² for population groups aged 1 year and older (EFSA NDA Panel, 2019a). Considering the maximum sodium content in the NF of 4.2%, the intake of sodium from the NF is expected to represent up to 0.9% (up to 10 mg sodium/day) of the sodium intake of 1.1 g/day considered as safe and adequate for toddlers (1–3 years). For other children and adults, the intake of sodium from the NF can represent up to 0.1% of the sodium intake levels and is considered safe and adequate for these age groups.

As for infants up to the age of 6 months consuming IF, the maximum sodium intake from the NF would be approximately 17 mg/day considering a daily intake of IF of 260 mL/kg bw. This corresponds to about 14% of the daily sodium intake of exclusively breastfed infants (120 mg sodium/day during the first 6 months; EFSA NDA Panel, 2019c).

For older infants aged 7–11 months, the Panel established an adequate intake (AI)¹³ of 200 mg/day (EFSA NDA Panel, 2019c). In this age group, the maximum sodium intake from the NF is estimated to be approximately 19 mg sodium/day, which corresponds to about 10% of the AI.

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 three toxicological studies on a mixture of HiMOs containing the NF, which were conducted in compliance with OECD (Organisation for Economic Co-operation and Development) principles of Good Laboratory Practices (GLP) (OECD, 1998) and in accordance with the OECD test guidelines (TG) 471 (OECD, 1997), 487 (OECD, 2016) and 408 (OECD, 2018). An additional preliminary *in vivo* repeated dose study was also carried out. These studies were conducted with a mixture of HiMOs composed by 2'-FL (47.1%), 3-FL (16.0%), LNT (23.7%), 3'-SL sodium salt (4.1%), 6'-SL sodium salt (4.0%) and other carbohydrates (5.1%). These studies, which were claimed proprietary by the applicant, are listed in Table 9.

An article on the assessment of the NF in the above-mentioned mixture of HiMOs, which describes the studies listed in Table 9, was provided (Parschat et al., 2020).

In addition, the applicant provided a publication where the toxicological evaluation of chemically synthesised 3'-SL has been described (Kim et al., 2018).

Table 9: List of toxicological studies with the NF (as component of the mixture of HiMOs)

Reference	Type of study	Test system	Dose
Unpublished Study, LPT No. 35908 (Parschat et al., 2020)	Bacterial reverse mutation test (GLP, OECD TG 471)	<i>Salmonella</i> Typhimurium TA98, TA100, TA102, TA1535 and TA1537	0.2–24.6 mg 3'-SL/plate (absence and presence of S9 mix)
Unpublished Study, LPT No. 35909 (Parschat et al., 2020)	<i>In vitro</i> mammalian cell micronucleus test in human peripheral blood lymphocytes (GLP, OECD TG 487)	Human peripheral blood lymphocytes	0.3–2.46 mg 3'-SL/mL for 4 or 24 h (absence and presence of S9 mix)
Unpublished study, LPT No. 35504 (Parschat et al., 2020)	7-day repeated dose oral toxicity study (pilot study)	SD rats (females only)	Dietary exposure ranging from 6.7 to 13.7 g/kg bw/day (mean 3'-SL intake: 0.27–0.56 g/kg bw per day)

¹² These levels of sodium intake are called 'safe' because it takes account of the evidence describing the relationship between sodium intake and CVD risk in the general population and 'adequate' in line with the definition of an adequate intake.

¹³ 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.

Reference	Type of study	Test system	Dose
Unpublished Study, LPT No. 35907 (Parschat et al., 2020)	90-day repeated dose oral toxicity study (GLP, OECD TG 408, limit test)	SD rats	Overall dietary exposure to 3'-SL of 4.1% of the mixture (mean intake of 0.23 and 0.29 mg 3'-SL/kg bw per day in males and females, respectively)

The Panel also noted that in solution under acidic conditions, the NF will be hydrolysed to D-lactose and sialic acid (EFSA NDA Panel 2020a). The amount of sialic acid potentially formed at the maximum concentration and intake (i.e. infants at 95th percentile (Table 4)) would be 0.8 mg/kg bw, which is lower than the intake based on natural levels in human milk (i.e. 11 mg/kg bw (EFSA NDA Panel, 2017)). Another monosaccharide, N-acetyl-D-glucosamine, can be found in the NF at levels up to 1.5 mg/kg bw. It is also present in human milk at concentrations up to 1.5 g/mL (Chen et al., 2010). Under alkaline conditions, 3'-sialyllactose would also be formed although the content would remain very low (up to 2.5 mg/kg bw) and is considered not of concern (suggested use in infants to treat constipation is 2.5 mL syrup/day corresponding to approximately 1.65 g lactulose/day (250 mg/kg bw for an infant weighing 6.7 kg)).

3.10.1. Genotoxicity

The *in vitro* assessment of the mutagenic potential of the mixture of HiMOs containing the NF was performed with *S. Typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537, which were exposed to the mixture diluted in water at six different concentrations up to 600 mg mixture/plate, either in the presence or absence of liver microsomal fractions (S9). No reproducible or dose-related increases in revertant colony numbers over control counts were observed with any of the strains following exposure to the mixture of HiMOs at any concentration (irrespective of the presence or absence of S9). No evidence of toxicity was obtained following exposure to the mixture of HiMOs. Therefore, the mixture of HiMOs was shown to be non-mutagenic at concentrations up to 600 mg/plate (corresponding to about 25 mg/plate of 3'-SL), in the absence or presence of metabolic activation.

In the *in vitro* mammalian cell micronucleus test, five concentrations of the mixture of HiMOs up to 60 mg/mL were tested in cultured human peripheral blood lymphocytes in the presence or absence of metabolic activation (S9 fraction). No statistically significant increases in the number of binucleated cells containing micronuclei both after 4-h treatment in the presence of S9 mix or following 24-h treatment in the absence of S9 were recorded. The mixture of HiMOs did not show any evidence of clastogenicity or aneugenicity in the absence and presence of metabolic activation up to the highest concentration of 60 mg/mL (corresponding to about 2.5 mg 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. Repeated dose toxicity studies

The applicant provided a 7-day repeated dose pilot toxicity study, where two groups of five CrI:CD (SD) female rats were given *ad libitum* a standard diet with and without 10% (w/w) of the mixture of HiMOs. The calculated intake of the mixture ranged from 6.7 to 13.7 g/kg bw per day, corresponding to a 3'-SL intake of 0.27–0.56 g/kg bw per day. There were no deaths or any relevant variations in clinical signs, food consumption or body weight. Clinical pathology investigations and post-mortem observations were not performed.

In the 90-day study (limit test) groups of 10 CrI:CD(SD) rats/sex were given *ad libitum* a standard diet with or without 10% (w/w) of the mixture of HiMOs (same composition as in the pilot study). The mean intake of the test item ranged from 5.01 to 6.88 g/kg bw per day (mean of 5.67) for the male animals and from 6.26 to 7.91 g/kg bw per day (mean of 6.97) for the female animals. The corresponding mean daily intake of 3'-SL has been calculated as 232 and 286 mg/kg bw for male and female rats, respectively.

There were no deaths in the course of the study and no treatment-related clinical signs were observed in any rats. Episodes of increased or decreased food consumption were recorded in treated males in comparison to the control group. Body weight and body weight gain were not affected by the treatment. Some statistically significant changes were noted: reduced spontaneous motility was

observed in treated male rats in the absence of any other change in functional observation tests and a slight increase in body temperature was noted in female rats.

Variations in haematological (decrease of neutrophils (–11%) in females) and clinical chemistry parameters (decrease in proteins (–9%, both albumin and globulin and increase in albumin/globulin ratio) and alanine aminotransferase (–24%)), increase in urea (+16%) in females) and urinalysis (decrease in specific gravity (–1%) in females) were recorded. A decrease in absolute brain weight (–2.9%) in treated males and relative kidneys weight (about –10%) in female rats was also noted. In male animals at histological examination, a small increase in the incidence and magnitude of hepatocellular lipid content in periportal areas was recorded. No other gross or histopathologic findings in treated rats were noted.

The changes observed were of low magnitude and limited to only one sex and they are overall considered by the Panel as not biologically relevant.

The Panel considers that no adverse effects were observed in this study at the tested dose corresponding to 0.23 g 3'-SL/kg bw per day.

3.10.3. Human data

No human intervention studies conducted with 3'-SL alone have been provided by the applicant.

The applicant provided instead a clinical study report corresponding to a double-blind, controlled, randomised interventional study conducted in infants with IF containing the mixture of HiMOs described in Section 3.10.2 (5.75 g/L, corresponding to about 0.23 g/L of 3'-SL). The main goal of the study was to investigate the suitability of the HiMO mixture – IF to support normal physical growth (evaluated per weight gain), in comparison with standard IF and breastfed infants. The study was conducted over a 112-day period in a total of 341 subjects. Secondary endpoints of tolerability (e.g. stool frequency and consistency, digestive tolerance) were also assessed. 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). The Panel considers the information provided by the applicant as supportive for the assessment of 3'-SL.

The applicant also provided references to clinical studies (Cooper et al., 2017; Meli et al., 2014; Radke et al., 2017; Simeoni et al., 2016) conducted in infants where tolerability of IF supplemented with bovine milk-derived oligosaccharides up to 10 g/L formula (including unspecified amount of 3'-SL) was assessed. The Panel considers that the test materials used in these studies are not representative of the NF and that therefore the results are of limited value for the safety assessment of the NF.

3.11. Allergenicity

The protein content in the NF is low ($\leq 0.01\%$) as indicated in the specifications (Table 3).

The applicant provided evidence for the absence of viable cells of production and optional degradation strains in the NF.

The applicant did not identify any allergenic potential of introduced proteins as a result of the genetic modification of the *E. coli* BL21 (DE3) host 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), by using 'higher than 35% identity in a sliding window of 80 amino acids' as the criterion.

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 sodium salt of the HiMO 3'-SL, but it also contains D-lactose, sialic acid, N-acetyl-D-glucosamine and 3'-sialyllactulose, and a small fraction of other related saccharides. The NF is obtained by fermentation with two genetically modified strains of *E. coli* BL21 (DE3), the production strain and the optional degradation strain.

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

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

3'-SL concentrations are relatively low in human milk and the most abundant acidic HMO is its constitutional isomer 6'-SL. The Panel notes that a safety assessment of 3'-SL, when produced with a derivative strain of *E. coli* K-12 DH1, has been carried out by EFSA (EFSA NDA Panel, 2020a) and 3'-SL sodium salt is included in the Union list of authorised NFs. The Panel also notes that other HiMOs (LNnT and LNT) produced by fermentation with derivative strains of the same host strain *E. coli* BL21 (DE3) have been recently assessed with a positive outcome (EFSA NDA Panel, 2020c, 2022c).

The submitted toxicity studies did not raise safety concerns. The Panel considers that no adverse effects were observed in the subchronic toxicity study at the tested dose corresponding to a daily intake of 0.23 g 3'-SL/kg bw.

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

The maximum daily intake of 3'-SL in FS for individuals above 3 years of age (i.e. 700 mg/day) or in infants and young children (i.e. 230 mg/day) does not exceed the highest intake level of 3'-SL 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 are consumed on the same day. The Panel similarly notes that infants and young children should not consume human milk and the FS on the same day.

Additional sources for the oligosaccharides contained in the NF are cow milk and milk-derived products. However, in light of the recent evidence revising the concentrations of 3'-SL in human milk, the contribution from consumption of cow milk and milk-derived products is small.

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 of breastfed infants and the estimated intake as NF.

5. Conclusions

The Panel concludes that the NF, which is composed of 3'-SL sodium salt 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 MS, NMR spectroscopy and HPAEC-PAD; (ii) toxicological information, including *in vitro* genotoxicity studies, subacute and subchronic toxicity studies (Table 9); (iii) description and certificates of deposition of the genetically modified production and optional degradation strains, qPCR detection system and method validation reports for the production and optional degradation strains; (iv) method validation reports for the determination of 3'-SL and carbohydrate by-products in the NF using HPAEC-PAD; (v) evaluation of nutritional suitability and tolerability of a HiMO mixture-containing IF for term infants (clinical study report).

6. Steps taken by EFSA

- 1) On 18 December 2020, 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. Ref. Ares (2020)7728598.
- 2) On 18 December 2020, a valid application on 3'-SL sodium salt, which was submitted by Chr. Hansen A/S, was made available to EFSA by the European Commission through the Commission e-submission portal (NF 2020/1794) and the scientific evaluation procedure was initiated.

- 3) On 14 April 2021, EFSA received a letter from the European Commission with the updated request for a scientific opinion on the safety of 3'-Sialyllactose (3'-SL) sodium salt. Ref. Ares (2021)2527652.
- 4) On 14 April 2021, a valid application on 3'-SL sodium salt was made available to EFSA by the European Commission through the Commission e-submission portal and the scientific evaluation procedure was restarted.
- 5) On 22 April 2021, EFSA requested the applicant to provide additional information to accompany the application and the scientific evaluation was suspended.
- 6) On 27 January 2022, additional information was provided by the applicant through the Commission e-submission portal and the scientific evaluation was restarted.
- 7) During its meeting on 29 April 2022, the NDA Panel, having evaluated the data, adopted a scientific opinion on the safety of 3'-SL sodium salt as a NF pursuant to Regulation (EU) 2015/2283.

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Abbreviations

1D	Mono-dimensional
2D	Two-dimensional
2'-FL	2'-Fucosyllactose
3-FL	3-Fucosyllactose
3'-SL	3'-Sialyllactose
6'-SL	6'-Sialyllactose
ADME	Absorption, Distribution, Metabolism and Excretion
AI	Adequate intake
ASU	Official collection of analysis methods according to § 64 of the German Food and Feed Code (LFGB)
bw	Body weight
CAS	Chemical Abstracts Service
CFU	Colony forming unit
CID	Collision induced decay
COSY	Correlated spectroscopy
CrI:CD(SD) rats	Charles River Laboratories: Cesaerean-derived (Sprague Dawley) rats
DEPT	Distortionless enhancement by polarisation transfer
DFL	Difucosyllactose
DIN	German Institute for Standardisation e. V.
DM	Dry matter
DNA	Deoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
EN	European norm
EU	Endotoxin unit
FEEDAP	EFSA Panel on Additives and Products or Substances used in Animal Feed
FOF	Follow-on formula
FoodEx2	EFSA standardised food classification and description system
FSSC 22000	Food Safety System Certification 22000
FS	Food supplements
FSMP	Food for special medical purposes
Gal	D-Galactose
Glc	D-Glucose
GLP	Good Laboratory Practice

GMO	EFSA Panel on Genetically Modified Organisms
GMP	Good Manufacturing Practice
GRAS	Generally Recognised As Safe
GRN	GRAS Notice
HACCP	Hazard Analysis Critical Control Points
HMBC	Heteronuclear multiple-bond correlation
HiMO	Human-identical milk oligosaccharide
HMO	Human milk oligosaccharide
HPAEC-PAD	High-performance anion-exchange chromatography – pulsed amperometric detection
HPLC-ESI	High-performance liquid chromatography – electrospray ionisation
HSQC	Heteronuclear single quantum correlation
IAC-HPLC-FD	Immunoaffinity chromatography – high-performance liquid chromatography – fluorescence detector
ICP-MS	Inductively coupled plasma – mass spectrometry
IF	Infant formula
ISO	International Organisation for Standardisation
IUPAC	International Union of Pure and Applied Chemistry
LFGB	German Food and Feed Code
LNT	Lacto-N-tetraose
LNnT	Lacto-N-neotetraose
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NANA, Neu5Ac	N-acetyl-D-neuraminic acid
ND	Not detected
NDA	EFSA Panel on Nutrition, Novel Foods and Food Allergens
NF	Novel food
NMR	Nuclear magnetic resonance spectroscopy
OECD	Organisation for Economic Co-operation and Development
Ph. Eur.	European Pharmacopeia
qPCR	Quantitative polymerase chain reaction
RH	Relative humidity
RNA	Ribonucleic acid
SD rats	Sprague Dawley rats
TG	Test guidelines
TOCSY	Total correlation spectroscopy
TS	Technical specification
US	United States
US FDA	US Food and Drug Administration
w/v	weight per volume
w/w	weight per weight

Annex A – Dietary exposure estimates to the Novel Food for each population group from each EU dietary survey

Information provided in this Annex is shown in an Excel file (downloadable at <https://efsa.onlinelibrary.wiley.com/doi/10.2903/j.efsa.7331#support-information-section>).