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

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SCIENTIFIC OPINION

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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

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Abstract

Following a request from the European Commission, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked to deliver an opinion on lacto-N-tetraose (LNT) as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The NF is a powdered mixture mainly composed of the human-identical milk oligosaccharide (HiMO) LNT, but it also contains D-lactose, lacto-N-triose II and *para*-lacto-N-hexaose, and a small fraction of other related saccharides. 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 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 LNT from the NF at the maximum proposed use levels does not exceed the intake level of naturally occurring LNT in breastfed infants on a body weight basis. The intake of LNT 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 LNT is also considered of no safety concern. Food supplements are not intended to be used if other foods with added LNT or human milk are consumed on the same day. The Panel concludes that the NF is safe under the proposed conditions of use.

Keywords: lacto-N-tetraose, LNT, human milk oligosaccharide, HMO, HiMO, novel food, safety

Requestor: European Commission

Question number: EFSA-Q-2020-00630

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1. Introduction

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

On 21 May 2020, the company Chr. Hansen A/S submitted a request to the Commission in accordance with Article 10 of Regulation (EU) 2015/2283[1] to place on the EU market, lacto-N-tetraose (LNT).

LNT is intended to be used in a number of foods, in food for special medical purposes (FSMP) as defined by Regulation (EU) No 609/2013[2] and in food supplements (FS) as defined in Directive 46/2002/EC[3].

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

In accordance with Article 10(3) of Regulation (EU) 2015/2283, the European Commission asks the European Food Safety Authority to provide a scientific opinion on LNT.

1.2. Additional information

LNT is included in the Union list of authorised NFs (Commission Implementing Regulation (EU) 2017/2470[4]) 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 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), LNT produced with a derivative strain of *E. coli* K-12 DH1 (EFSA NDA Panel, 2019b), 3′-sialyllactose (3′-SL) sodium salt (EFSA NDA Panel, 2020a), 6′-sialyllactose (6′-SL) sodium salt (EFSA NDA Panel, 2020b), LNnT (EFSA NDA Panel, 2020c), 3-fucosyllactose (3-FL) (EFSA NDA Panel, 2021) and 2′-FL/DFL mixture and LNT in FS for infants (EFSA NDA Panel, 2022).

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) 2017/2469[5].

A common and structured format on the presentation of NF applications is described in the EFSA guidance on the preparation and presentation of an 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.

[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. OJ L 327, 11.12.2015, p. 1–22.

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

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

[4] Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, p. 72–201.

[5] 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.

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

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 FSMP are laid down in Regulation (EU) No 609/2013 and, respectively, in Commission Delegated Regulation (EU) 2016/128[6] (FSMP), and in Commission Delegated Regulation (EU) 2016/127[7] (as regards the specific compositional and information requirements for infant formula (IF) and follow-on formula (FOF) and as regards requirements on information relating to infant and young child feeding).

This assessment concerns only the risks that might be associated with consumption of the NF under the proposed conditions of use and is not an assessment of the efficacy of the NF with regard to any claimed benefit. 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, which is the subject of the application, contains LNT as primary constituent (≥ 75% w/w dry matter (DM)). LNT is a neutral core oligosaccharide and one of the most abundant oligosaccharides present in human milk (HMOs). The Panel notes that although LNT is the major component of the NF, related substances, namely lacto-N-triose II, *para*-lacto-N-hexaose, D-lactose 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.

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/EC. The target population is the general population.

LNT produced with a derivative strain of *E. coli* K-12 DH1 has been previously assessed by EFSA as a NF with a positive outcome (EFSA NDA Panel, 2019b). 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).

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 LNT (≥ 75% w/w DM), but it also contains D-lactose (≤ 5% w/w DM), D-galactose and D-glucose (≤ 5% w/w DM, sum of both), *para*-lacto-N-hexaose (≤ 5% w/w DM), lacto-N-triose II (≤ 5% w/w DM) and a small fraction of other related saccharides (sum of other carbohydrates ≤ 15% w/w DM). It is produced by fermentation with two

[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.

genetically modified strains of *E. coli* BL21 (DE3), the production strain and the optional degradation strain. LNT is a tetrasaccharide consisting of D-galactose linked through a β-(1-3) bond to N-acetyl-D-glucosamine (GlcNAc), linked through a β-(1-3) bond to D-galactose, linked through a β-(1-4) bond to the reducing end D-glucose, in its α- and β-anomeric forms (Table 1 and Figure 1). LNT is a regioisomer of LNnT, which contains the same monosaccharide moieties as those present in LNT but with the linkage between the terminal D-galactose and GlcNAc being β-(1-4) rather than β-(1-3).

Table 1: Chemical identity of LNT

Chemical substance	
Chemical (IUPAC) name	β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Common name	Lacto-N-tetraose; Gal-(β1-3)-GlcNAc-(β1-3)-Gal-(β1-4)-Glc
Abbreviations, other names	LNT, Chr. Hansen LNT
CAS Number	14116-68-8
Molecular formula	$C_{26}H_{45}NO_{21}$
Molecular weight	707.63 Da

The molecular structure of LNT 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, which allowed to differentiate between LNT (β-1-3) and LNnT (β-1-4). The mass fragmentation pattern is consistent with that reported in the literature (Chai et al., 2001; Pfenninger et al., 2002).

The identity of LNT 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 LNT has been confirmed by mono-dimensional (1D) nuclear magnetic resonance (NMR) spectroscopy including ^{1}H, ^{13}C and ^{13}C-DEPT-90 (distortionless enhancement by polarisation transfer) spectra, and two-dimensional (2D) NMR spectroscopy, including ^{1}H-^{1}H COSY (correlated spectroscopy), ^{1}H-^{13}C HSQC (heteronuclear single quantum correlation), ^{1}H-^{13}C HMBC (heteronuclear multiple-bond correlation) and ^{1}H-^{13}C HSQC-TOCSY (heteronuclear single quantum correlation total correlation spectroscopy) spectra. The coupling constants ($J_{H1,H2}$) indicate that the pyranose ring configurations are β for all monosaccharide units, except for the reducing end D-glucose, where the two anomeric forms in solution are in equilibrium. The presence of the β-(1-3) linkage between the terminal D-glucose and N-acetyl-D-glucosamine in LNT, indicating the steric proximity of the H-1 atom of the terminal D-glucose unit and the H-3 atom of the N-acetyl-D-glucosamine unit, has been confirmed by 2D NMR spectra. Correlations in the HSQC spectrum were identified and assigned based on the chemical shifts previously reported for LNT (Strecker et al., 1989).

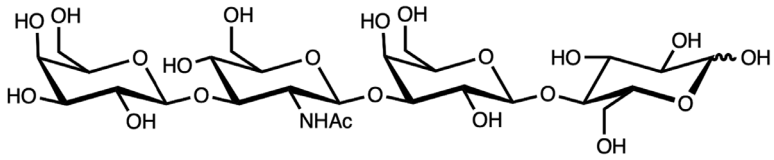

Figure 1: Chemical structure of LNT (EFSA NDA Panel, 2019b)

The LNT produced by the microbial fermentation described has been shown to be chemically and structurally identical to a commercially available LNT 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) JBT-PS-LNT and the optional 'degradation strain' *E. coli* BL21 (DE3) JBT-DS-LN(n)T. The

production strain has been modified to effectively synthesise LNT, 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 LNT 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 LNT. 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 LNT is spray-dried to obtain a powder form.

The production and the optional degradation strains *E. coli* BL21 (DE3) JBT-PS-LNT and *E. coli* BL21 (DE3) JBT-DS-LN(n)T, respectively, are genetically modified derivatives of the host strain *E. coli* BL21 (DE3) (F^- *omp*T *hsd*S_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 *lac*UVA 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 two and four antimicrobial resistance genes introduced during the genetic modification of the production and the optional degradation strains, respectively. 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 LNT as primary ingredient (86.5% w/w DM[8]). The remainder is a mixture of substances[8,9] such as D-lactose (0.9% w/w DM), D-galactose and D-glucose (0.4% w/w DM, sum of both), *para*-lacto-N-hexaose (1.6% w/w DM) and lacto-N-triose II (1.2% w/w DM). In addition, the NF contains other carbohydrates individually present at low concentration (sum of other carbohydrates, 9.0% w/w DM[8,9]). D-Lactose is the most abundant component of human milk (≈ 7% w/v) and its monomers D-glucose and D-galactose are normal constituents of human milk. Lacto-N-triose II is a metabolic intermediate of LNT (and LNnT) biosynthesis and one of the products of LNT (or LNnT) hydrolysis (Kuhn et al., 1956), and as such occurs also naturally in human milk (Dabrowski et al., 1983; Hosomi and Takeya, 1988; Miwa et al., 2010). The hexasaccharide, *para*-lacto-N-hexaose is naturally present in human milk and firstly isolated and described by Yamashita et al. (1977).

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 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 five 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 five 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 five batches of the NF and the sum of other carbohydrates.

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

Parameter	Batch number					Method of analysis
	#1	#2	#3	#4	#5	
Composition						
LNT (% w/w DM)	89.8	84.8	81.6	83.5	92.7	HPAEC-PAD (validated internal method)[1]
Lacto-N-triose II (% w/w DM)	1.7	1.7	1.7	< LOQ	< LOQ	
D-Lactose (% w/w DM)	1.1	1.1	1.1	1.1	< LOQ	
para-lacto-N-hexaose (% w/w DM)	1.8	1.9	1.8	1.9	< LOQ	
D-Galactose and D-glucose[2] (% w/w DM)	0.5	0.5	0.5	< LOQ	0.3	
Sum of other carbohydrates (% w/w DM)	4.7	9.8	13.0	12.5	5.2	Calculation[3]
Protein (%)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	Nanoquant (modified Bradford)
Ash (%)	0.44	0.23	0.34	0.35	0.14	ASU L 06.00-4
Water (%)	5.5	5.4	5.5	5.6	5.8	Karl Fischer titration
Contaminants						
Arsenic[4] (mg/kg)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	ASU L 00.00-135: 2011-01 – ICP-MS
Cadmium[4] (mg/kg)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
Lead[4] (mg/kg)	< LOQ	< LOQ	0.016	< LOQ	< LOQ	
Mercury[4] (mg/kg)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	
Aflatoxin M1 (μg/kg)	< 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	20	< 10	< 10	ISO 4833-2
Yeast and mould (CFU/g)	< 10	< 10	< 10	< 10	< 10	ISO 21527-2: 2008-07
Enterobacteriaceae (CFU/g)	< 10	< 10	< 10	< 10	< 10	ISO 21528-2: 2019-05
Salmonella (in 25 g)	ND	ND	ND	ND	ND	DIN EN ISO 6579-1: 2017-07
Cronobacter spp. (in 10 g)	ND	ND	ND	ND	ND	ISO/TS 22964: 2017-04
Listeria monocytogenes (in 25 g)	ND	ND	–	ND	ND	DIN EN ISO 11290-1: 2017-09
Bacillus cereus (in 5 g)	ND	ND	–	ND	ND	ASU L 00.00-108: 2007-04
Endotoxins (EU/mg)	0.228	0.034	0.031	< 0.005	< 0.005	Ph. Eur. 2.6.14

'–': Not reported; 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 detection; ICP-MS: Inductively coupled plasma – mass spectrometry; ISO: International Organisation for Standardisation; LNT: Lacto-N-tetraose; LOQ: Limit of quantification; ND: Not detected; Ph. Eur.: European Pharmacopeia; TS: Technical specification.

1: LOQs: D-Lactose = 0.27% w/w DM; D-Galactose and D-glucose = 0.14% w/w DM; Lacto-N-triose II = 0.54% w/w DM; *para*-lacto-N-hexaose = 0.81% w/w DM.

2: D-Galactose and D-glucose peaks on the HPAEC-PAD chromatograms overlap at 3.7 min retention time.

3: Sum of other carbohydrates = 100 (% w/w DM) – LNT (% 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.

4: 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 LNT 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 LNT (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 LNT (expressed on a DM basis) remained relatively stable over the 156-week period under normal storage conditions (average content of 25.0% w/w DM), with an increase in the moisture content from 5.7% to 10.9%, which exceeds the specifications (≤ 9.0%). Over the 26-week storage under accelerated conditions, the content of LNT (expressed on a DM basis) remained relatively unchanged (average content of 24.9% w/w DM), although 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 8.2 ± 0.6% in the NF and 6.6 ± 1.2% in the HiMO mixture).

The applicant also referred to the stability studies included in the GRAS notification 833 (US FDA, 2019) on LNT produced with a derivative strain of *E. coli* K-12 DH1, as well as to the stability of the authorised NF for at least 24 months when stored at room temperature (EFSA NDA Panel, 2019b). The applicant proposed a 1-year shelf-life under ambient storage conditions for the NF.

The Panel considers that the data provided sufficient information with respect to the stability of the NF for one 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 LNT 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 LNT 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' (EFSA NDA Panel, 2019b). Moreover, LNnT, a constitutional isomer of LNT, has been demonstrated to be stable in various food matrices, including yoghurt, ready-to-drink flavoured milk and citrus fruit beverages (EFSA NDA Panel, 2015b).

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

Description: Lacto-N-tetraose (LNT) is a white- to ivory-coloured powder produced by microbial fermentation and further isolated, purified and concentrated.	
Source: Two genetically modified strains of *Escherichia coli* BL21 (DE3).	
Parameter	**Specification**
Composition	
LNT (% w/w DM)	≥ 75
Sum of other carbohydrates[1] (% w/w DM)	≤ 15
D-Lactose (% w/w DM)	≤ 5
Lacto-N-triose II (% w/w DM)	≤ 5
para-lacto-N-hexaose (% w/w DM)	≤ 5
D-Galactose and D-glucose[2] (% w/w DM)	≤ 5
Water (%)	≤ 9.0
Protein (%)	≤ 0.01
Ash (%)	≤ 1.0
Contaminants	
Arsenic (mg/kg)	≤ 0.2
Aflatoxin M1 (μg/kg)	≤ 0.025
Microbial parameters	
Standard plate count (CFU/g)	≤ 1,000
Yeast and mould (CFU/g)	≤ 100
Enterobacteriaceae (CFU/g)	≤ 10
Salmonella (in 25 g)	ND
Cronobacter (*Enterobacter*) *sakazakii* spp. (in 10 g)	ND
Endotoxins (EU/mg)	≤ 10

CFU: Colony forming unit; DM: Dry matter; EU: Endotoxin unit; LNT: Lacto-N-tetraose; ND: Not detected.

1: Sum of other carbohydrates = 100 (% w/w DM) – LNT (% 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.

2: D-Galactose and D-glucose peaks on the HPAEC-PAD chromatograms overlap at 3.7 min retention time.

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 not history of use of the NF. However, LNT produced by fermentation with a genetically modified strain of *E. coli* K-12 DH1 has been authorised as a NF in the European Union (Commission Implementing Regulation 2020/484[10]) to be added to IF, FOF and a variety of foods as well as FS, and has also been granted GRAS status (GRN 833) in the US (US FDA, 2019).

Other HiMOs, i.e. the constitutional isomer LNnT and 2'-FL, produced by the same applicant with derivative strains of *E. coli* BL21 (DE3) have been included in the Union list of authorised NFs ((EU) 2018/1023).

Oligosaccharides have also been detected in domestic farm animal milk, however at lower concentrations as compared to human milk (Urashima et al., 2012, 2013; Aldredge et al., 2013; Albrecht et al., 2014), with LNT concentrations of about 11 μg/mL being reported in bovine milk (Wang et al., 2020).

[10] Commission Implementing Regulation (EU) 2020/484 of 2 April 2020 authorising the placing on the market of lacto-N-tetraose 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 103, 3.4.2020, p. 3–90.

3.6.2. Intake of LNT 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, as 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). HMOs concentrations and composition vary across mothers and over the course of lactation. LNT belongs to the subfraction of 'neutral core' HMOs, and it is one of the most abundant HMOs along with 2'-FL, 6'-SL, lacto-N-fucopentaose I and LNnT.

Core HMOs such as LNT and LNnT are present in all human milk phenotypes (Erney et al., 2000; Kunz et al., 2017). The biological relevance of the structural difference between LNT and LNnT, i.e. type 1 vs. type 2 linkage between the terminal Gal and next to GlcNAc (and the respective ratio between the two forms) is not clear.

Several publications on HMO and LNT in human milk have been provided by the applicant. The amount of LNT in human milk, as for other HMOs, is genetically determined and also depends on the lactation period, with higher levels reported in colostrum (Coppa et al., 1999, 2001; Erney et al., 2000; Asakuma et al., 2008, 2011; Thurl et al., 2010, 2017; De Leoz et al., 2012; Galeotti et al., 2012, 2014; Spevacek et al., 2015; Austin et al., 2016; McGuire et al., 2017). Thurl et al. (2017) summarised the findings from 21 studies and reported that the mean concentration of LNT in milk from mothers who delivered at term ranged from 0.59 to 0.98 g/L (average 0.79 g/L). It was noted that the concentration of LNT was slightly more abundant (ranging from 0.39 to 1.68 g/L, average 1.04 g/L) in mothers who delivered pre-term. Other publications reported maximum concentrations in European human milks up to 3.3 (average 0.5–2.5 g/L; Austin et al., 2019) or 6.7 g LNT/L (average 0.5–1.2 g/L; Samuel et al., 2019). In a recent review (Soyyilmaz et al., 2021), a mean concentration of 0.74 g/L has been reported in mature milk with a maximum mean of 1.6 g LNT/L. Erney et al. (2001) reported an average concentration of 0.76 g/L (with a maximum concentration of 2.74 g/L) in pooled milk samples from different lactating phases. LNT was found in all samples collected from 264 mothers.

The daily intake levels of LNT from human milk for a 6.7 kg body weight (bw) infant (EFSA Scientific Committee, 2012) have been calculated (Table 4) considering the mean and the highest concentrations of LNT in human milk as reported by Erney et al. (2001) and also used in the previous LNT opinion (EFSA NDA Panel, 2019b), and 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).

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 LNT 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 0.76 g/L and 2.74 g/L LNT, respectively (Erney et al., 2001; EFSA NDA Panel, 2019b)

	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**
LNT	91	327	136	491

bw: body weight.

The Panel noted that although the main component of the NF is LNT, other fractions such as D-lactose, lacto-N-triose II and *para*-lacto-N-hexaose are present in different amounts. While D-lactose is the most represented molecule in human milk, less is known for the other two fractions lacto-N-triose II and *para*-lacto-N-hexaose (see Section 3.4). The concentration of these oligosaccharides in human milk has not been quantified.

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 FoodEx2[11] 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 4.6 g LNT for individuals above 3 years of age or at a maximum daily intake of 1.82 g LNT 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 intended by the applicant

FoodEx2 code	FoodEx2 level	Food category	Max use level[(a)] (mg LNT/100 g)
A03PZ	4	Infant formulae, powder	1,456[(b)]
A03QE	4	Infant formulae, liquid	182
A03QK	4	Follow-on formulae, powder	1,456[(b)]
A03QQ	4	Follow-on formulae, liquid	182
A03QZ	3	Cereals with an added high protein food which have to be reconstituted with water or other protein-free liquid	728[(b)]
A03QY	3	Simple cereals which have to be reconstituted with milk or other appropriate nutritious liquids	1,274[(b)]
A0BZF	3	Cereals with an added high protein food reconstituted	182
A0BZE	3	Simple cereals for infants and children, reconstituted	182
A03RA	3	Biscuits, rusks and cookies for children	182
A03RB	3	Pasta for children (dry, to be cooked)	182
A03RH	3	Ready-to-eat dairy-based meal for children	182
A03RP	3	Special food for children's growth	182
A03RN	3	Fruit and vegetable juices and nectars specific for infants and young children	182

(a): The proposed maximum use level for all food categories is 1.82 g/kg expressed as LNT and it would correspond to a maximum use level of 2.10 g/kg when expressed as NF (86.5% LNT, see Section 3.4).
(b): 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 LNT 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 LNT (1.82 g/L in IF), the high intake of LNT from IF alone is estimated to be 473 mg/kg bw per day.

The Panel notes that the anticipated daily intake of LNT from the consumption of IF does not exceed the estimated high daily intake of LNT of 491 mg/kg bw in breastfed infants (Table 4).

Anticipated intake of LNT from the proposed uses and use levels

EFSA performed an intake assessment of the anticipated daily intake of LNT based on the applicant's proposed uses and maximum proposed use levels (Table 5), using individual data from the

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

EFSA Comprehensive European Food Consumption Database (EFSA, 2011). The lowest and highest mean and 95th percentile anticipated daily intakes of LNT (on an mg/kg bw basis), among the EU dietary surveys, are presented in Table 6.

The estimated daily intake of LNT 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 LNT 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	42	185	157	403
Young children[(c)]	1 to < 3	3	61	25	153
Other children	3 to < 10	0	3	0	20
Adolescents	10 to < 18	0	1	0	5
Adults[(d)]	≥ 18	0	0	0	2

(a): Intakes were assessed for all EU dietary surveys available in the food comprehensive database on 17 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 17 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 anticipated daily intake of LNT from the proposed uses and use levels does not exceed the estimated high daily intake of LNT of 491 mg/kg bw in breastfed infants (Table 4).

3.7.4. Anticipated intake of LNT from FS

The applicant has proposed a maximum daily intake of LNT of 4.6 g in FS for individuals above 3 years of age or a maximum level of 1.82 g/day when intended for infants (up to 11 months) or young children (12–35 months).

Table 7: Use of LNT 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	1,820	364
Young children[(c)]	1 to < 3	12	1,820	152
Other children	3 to < 10	23.1	4,600	199
Young adolescents	10 to < 14	43.4	4,600	106
Old adolescents	14 to < 18	61.3	4,600	75
Adults	≥ 18	70	4,600	66

(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 LNT from the use of the NF in FS (i.e. from 1.82 to 4.6 g/day) for any population category (Table 7) does not exceed the estimated high daily intake of LNT of 491 mg/kg bw in breastfed infants (Table 4).

According to the applicant, FS are not intended to be used if other foods with added LNT are consumed on the same day. The Panel similarly notes that in infants and young children also human milk should not be consumed.

3.7.5. Combined intake from the NF and other sources

The Panel notes that LNT 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)[4].

The combined daily intake of LNT 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 daily intake of LNT from already authorised uses and the currently proposed uses is higher (563 mg/kg bw, Table 8) than the estimated daily intake based on only the currently proposed uses and use levels (403 mg/kg bw, Table 6).

Table 8: Intake estimate resulting from the combined uses of LNT 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	66	235	172	563
Young children[(c)]	1 to <3	44	126	124	351
Other children	3 to <10	14	73	37	182
Adolescents	10 to <18	5	19	20	47
Adults[(d)]	≥18	8	13	29	31

(a): Intakes were assessed for all EU dietary surveys available in the food comprehensive database on 17 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 17 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 from the combined exposure (i.e. 563 mg/kg bw; Table 8) from the maximum authorised and proposed uses, is higher than the estimated daily intake from the authorised uses alone (i.e. 534 mg/kg bw; EFSA NDA Panel, 2019b), and approximately 15% above the high estimate for LNT daily intake in breastfed infants (i.e. 491 mg/kg bw; Table 4).

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

No ADME data have been provided for the NF.

As reported in previous EFSA opinions (EFSA NDA Panel, 2014, 2019a,b, 2020a,b, 2021), HMOs, including LNT, are considered 'non-digestible oligosaccharides' since they do not undergo any significant digestion in the upper gastrointestinal tract (Brand-Miller et al., 1995, 1998; Engfer et al., 2000; Chaturvedi et al., 2001; Gnoth 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 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; Gnoth et al., 2000; Goehring et al., 2014; Vazquez et al., 2017; EFSA NDA Panel, 2019b).

Specifically, for LNT, it has also been observed that breastfed infants may receive approximately 50–100 mg for each suckling and that an amount up to 3 mg in a day is found in the urine (Rudloff and Kunz, 2012; Rudloff et al., 2012).

In addition, it has been demonstrated for LNnT, a constitutional isomer of LNT, and few other HMOs, that absorption and urinary excretion also occur in the rat (Vazquez et al., 2017).

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 LNT, 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, 2019b).

3.9. Nutritional information

The NF is mainly composed by the non-digestible oligosaccharide LNT.

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

3.10. Toxicological information

The applicant provided three toxicological studies on 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 also provided a publication where the toxicological evaluation of LNnT, a constitutional isomer of LNT, has been described (Coulet et al., 2013) and made reference to the assessment performed on 2'-FL and LNnT (EFSA NDA Panel, 2015a,b).

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)	*Salmonella* Typhimurium TA98, TA100, TA102, TA1535 and TA1537	1.2–142.2 mg LNT/plate (absence and presence of S9 mix)
Unpublished Study, LPT No. 35909 (Parschat et al., 2020)	*In vitro* mammalian cell micronucleus test in human peripheral blood lymphocytes (GLP, OECD TG 487)	Human peripheral blood lymphocytes	1.8–14.2 mg LNT/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 per day (mean LNT intake: 1.59–3.25 g/kg bw per day)
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 LNT of 23.7% of the mixture (mean intake of 1.34 and 1.65 g LNT/kg bw per day in males and females, respectively)

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 NF 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 142 mg/plate of LNT), 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 increase 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 was 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 14 mg LNT/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 Crl:CD(SD) female rats were given *ad libitum* a standard diet with and without 10% of a mixture of HiMOs. The calculated intake of the mixture ranged from 6.7 to 13.7 g/kg bw per day, corresponding to a LNT intake of 1.59–3.25 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 Crl:CD(SD) rats/sex were given *ad libitum* a standard diet with or without 10% of the HiMO mixture (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 LNT has been calculated as 1.34 and 1.65 g/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), clinical chemistry parameters (decrease in proteins (9%, both albumin and globulin, and increase in albumin/globulin ratio) and alanine aminotransferase (24%), and 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 1.34 g LNT/kg bw per day.

3.10.3. Human data

No human intervention studies with LNT alone have been provided by the applicant.

Reference was also made by the applicant to studies conducted with the chemically synthesised constitutional isomer LNnT in adults and infants, and to the fact that data were overall sufficient to conclude about the safety of its use under the proposed conditions (EFSA NDA Panel, 2015b).

The Panel considers the information provided by the applicant as supportive for the assessment of LNT.

3.11. Allergenicity

The protein content in the NF is low (≤ 0.01%), with the batch-to-batch analysis resulting in protein levels below the limit of quantification (0.001%). The applicant provided evidence for the absence of viable cells of production and the 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 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), 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 HiMO LNT, but it also contains D-lactose, lacto-N-triose II and *para*-lacto-N-hexaose, 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 is the general population.

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

The Panel notes that a safety assessment of LNT, when produced by a derivative strain of *E. coli* K-12 DH1, has previously been carried out by EFSA (EFSA NDA Panel, 2019b) and LNT is included in the Union list of authorised NFs. The Panel also notes that another HiMO (the constitutional isomer LNnT) produced by fermentation with derivative strains of the same host *E. coli* BL21 (DE3) strain has been recently assessed with a positive outcome (EFSA NDA Panel, 2020c).

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 dose corresponding to a daily intake of 1.34 g LNT/kg bw.

The Panel notes that the anticipated daily intake of LNT from the consumption of IF (only), in infants up to 16 weeks of age, does not exceed the highest estimated daily intake level of LNT in breastfed infants on a body weight basis. The anticipated daily intake of LNT from the proposed uses at their respective maximum use levels in all population groups also does not exceed the highest daily intake level of LNT in breastfed infants on a body weight basis. However, when the combined intake with the already authorised uses of LNT is considered, an exceedance (15%) of the natural intake in infants is noted at the 95th percentile.

Considering that the exposure of LNT from human milk is only exceeded in 1 out of 12 dietary surveys included in the EFSA Food Consumption Database (Table 6 and Excel file annexed under 'supporting information', with yoghurt as main contributor) and the conservative assumption underlying this type of intake assessment (in particular, assuming that the NF is added at the maximum proposed use levels to all the proposed food categories consumed by infants), the Panel considers that it is unlikely that the LNT intake in infants would exceed the high LNT intake levels in breastfed infants.

The maximum daily intake in LNT in FS for individuals above 3 years of age (i.e. 4.6 g/day) or for infants and young children (i.e. 1.82 g/day) also does not exceed the highest intake level of LNT in breastfed infants per kg bw. The applicant stated that FS are not intended to be used if other foods with added LNT (as well as human milk for infants and young children) are consumed on the same day.

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

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 in breastfed infants and the estimated intake as NF.

5. Conclusions

The Panel concludes that the NF, which is composed of LNT 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 of the genetically modified LNT and LNnT production and optional degradation strains, qPCR detection system and method validation reports for the LNT production and optional degradation strains, certificates of

deposition of the LNT production and optional degradation strains, and genome sequence of the parental strain *E. coli* BL21 (DE3); (iv) method validation reports for the determination of LNT and carbohydrate by-products in the NF using HPAEC-PAD.

6. Steps taken by EFSA

1) On 27 January 2021 EFSA received a letter from the European Commission with the request for a scientific opinion on the safety of lacto-N-tetraose as a NF pursuant to Regulation (EU) 2015/2283. Ref. Ares (2021) 673184.
2) On 27 January 2021, a valid application on lacto-N-tetraose, which was submitted by Chr. Hansen A/S, was made available to EFSA by the European Commission through the Commission e-submission portal (NF 2020/1809) and the scientific evaluation procedure was initiated.
3) On 07 April 2021, EFSA received a letter from the European Commission with the updated request for a scientific opinion on the safety of lacto-N-tetraose. Ref. Ares(2021)848521.
4) On 07 April 2021, a valid application on lacto-N-tetraose was made available to EFSA by the European Commission through the Commission e-submission portal and the scientific evaluation procedure was restarted.
5) On 12 May 2021, EFSA requested the applicant to provide additional information to accompany the application and the scientific evaluation was suspended.
6) On 05 November 2021, additional information was provided by the applicant through the Commission e-submission portal and the scientific evaluation was restarted.
7) During its meeting on 23 March 2022, the NDA Panel, having evaluated the data, adopted a scientific opinion on the safety of lacto-N-tetraose 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′-Siayllactose
6′-SL	6′-Siayllactose
ADME	Absorption, Distribution, Metabolism and Excretion
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
Crl:CD(SD) rats	Charles River Laboratories: Caesarean-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
DS	Danish standard
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
FS	Food supplements
FSMP	Food for special medical purposes
FSSC 22000	Food Safety System Certification 22000
Gal	D-Galactose
Glc	D-Glucose
GlcNAc	N-acetyl-D-glucosamine
GLP	Good Laboratory Practice
GMO	EFSA Panel on Genetic 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 detection
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	N-acetyl-D-neuraminic acid
ND	Not detected
NDA	EFSA Panel on Nutrition, Novel Foods and Food Allergens
NF	Novel food
NMR	Nuclear magnetic resonance spectroscopy
NOAEL	No observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
Ph. Eur.	European Pharmacopeia
qPCR	Quantitative polymerase chain reaction
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/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.7242#support-information-section).