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Safety of lacto-*N*-neotetraose as a novel food ingredient pursuant to Regulation (EC) No 258/97

EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)

Abstract

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver an opinion on lacto-*N*-neotetraose as a novel food ingredient (NFI) submitted pursuant to Regulation (EC) No 258/97 of the European Parliament and of the Council, taking into account the comments and objections of a scientific nature raised by Member States. Lacto-*N*-neotetraose (LNnT) is a synthetic tetrasaccharide, which is intended to be used in infant and follow-on formulae, foods for special medical purposes for infants and young children and other foods for infants and young children, as well as in foods or food supplements for adults. The information provided on the potential mutagenicity of LNnT does not raise safety concerns as regards the genotoxicity of this NFI. Based on the observations from a sub-chronic 90-day toxicity study in rats, the Panel considers that the no observed adverse effect level is 2 500 mg/kg body weight per day. The applicant provided a double-blind, randomised, controlled clinical trial on the effects of LNnT consumed in combination with another oligosaccharide (2'-*O*-fucosyllactose (2'-FL)) in infants. The Panel concludes that LNnT is safe for infants (up to one year of age) when added to infant and follow-on formulae, in combination with 2'-FL, at concentrations up to 0.6 g/L of LNnT and up to 1.2 g/L of 2'-FL, at a ratio of 1:2 in the reconstituted formulae; is safe for young children (older than one year of age) when added to follow-on and young-child formulae, at concentrations up to 0.6 g/L of LNnT (alone or in combination with 2'-FL, at concentrations up to 1.2 g/L, at a ratio of 1:2). The Panel also concludes that LNnT is safe when added to other foods at the uses and use levels proposed by the applicant.

Keywords: lacto-*N*-neotetraose, LNnT, synthetic oligosaccharide, novel food ingredient, safety

Requestor: European Commission following an application by Glycom A/S

Question number: EFSA-Q-2014-00862

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Summary

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver an opinion on lacto-*N*-neotetraose as a novel food ingredient submitted pursuant to Regulation (EC) No 258/97 of the European Parliament and of the Council, taking into account the comments and objections of a scientific nature raised by Member States. The assessment follows the methodology set out in Commission Recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients (NFIs) and the preparation of initial assessment reports under Regulation (EC) No 258/97. The assessment is based on the data supplied in the original application, the initial assessment by the competent authority of Ireland, the concerns and objections of the other Member States and the responses of the applicant.

Lacto-*N*-neotetraose (LNnT) is a synthetic tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose and D-glucose, which is produced by using D-lactose as a starting raw material. The NFI is intended by the applicant to be used in infant and follow-on formulae, foods for special medical purposes for infants and young children, and other foods for infants and young children. The NFI is also intended to be used in foods or food supplements for adults. The Panel considers that the information provided on the specifications, the data from batch testing and the production process do not raise safety concerns.

Based on food consumption data of EFSA for infants and the use level of the NFI (0.6 g/L) proposed by the applicant, LNnT intakes from infant formula would result in about 637 mg per day and 104 mg/kg body weight, respectively, at the 95th percentile for a 3-month-old infant weighing 6 kg. According to the intake estimate provided by the applicant based on individual consumption data from the UK, LNnT intakes from infant formula could lead to an intake of about 330 mg/kg body weight per day at the 95th percentile for infants aged 4 to 6 months.

For other population groups, the applicant estimated in a tiered approach, intakes by using the EFSA Food Additive Intake Model tool based on summary statistics of consumption data of the EFSA Comprehensive Food Consumption Database. For a refined intake estimate, the applicant used individual consumption data recorded by the UK National Diet and Nutrition Survey for the years 2008–2010.

Considering that LNnT is a naturally occurring tetrasaccharide that is present in human milk, the history of human exposure to LNnT is limited primarily to that of breast-fed infants. The Panel notes that the proposed maximum intake level of the NFI by infants is within the range of intake levels from breast milk reported in the literature. Taking into account that there are no indications that the absorption of the NFI may differ from that of LNnT from breast milk, the Panel considers that the limited amount of NFI that could be absorbed does not raise safety concerns.

The applicant provided three studies on the potential mutagenicity of LNnT (a bacterial reverse mutation assay, an *in vitro* mammalian cell gene mutation test and an *in vitro* micronucleus assay). The Panel considers that the information provided does not raise safety concerns as regards the genotoxicity of the NFI.

Based on the observations from a sub-chronic 90-day toxicity study in rats, the Panel considers that the no observed adverse effect level is 2 500 mg/kg body weight per day.

The applicant provided an unpublished study report on a double-blind, randomised, controlled clinical trial on the effects of a combination of two oligosaccharides, LNnT and 2′-*O*-fucosyllactose (2′-FL), which are both under safety assessment as NFIs, in infants. This study reported that the weight gain of infants who consumed the formula with added LNnT and 2′-FL was not inferior to the weight gain of infants who were fed the control formula up to four months of age. The results on stool endpoints and the reported changes in the composition of microbiota in infants consuming the combination of the two oligosaccharides do not raise safety concerns for the studied combination. The Panel considers that this study can be used to draw conclusions on the safety of this combination at the concentrations tested (i.e. 0.5–0.6 g/L of LNnT and 1.0–1.2 g/L of 2′-FL for reconstituted formula).

Noting that complementary feeding of infants and young children results in an increasingly diversified diet, the Panel considers that the potential impact of the addition of LNnT alone (without 2′-FL) or in

combination with 2'-FL on the development of the microbiota diminishes with the proportion of complementary feeding and with age. The Panel therefore considers that LNnT alone or in combination with 2'-FL added at the proposed concentration in foods intended for children older than one year of age, does not raise safety concerns.

The applicant also provided an unpublished study report on a placebo-controlled, double-blind, parallel, dose-response trial, in healthy adults who were randomised to consume either LNnT or 2'-FL, alone or in different combinations, or placebo for two weeks. This study showed that participants who consumed daily either 20 g or 10 g of LNnT reported a significant increase in passing gas compared with the placebo group, while, for subjects consuming 5 g of LNnT per day, this effect was not observed. Considering the highest estimated 95th percentile daily intake of LNnT at 3.3 g and 53 mg/kg body weight, respectively, in women of child-bearing age plus the maximum intended daily intake from food supplements of 1.5 g, LNnT intakes in this population group could reach up to 4.8 g per day.

The Panel concludes that the NFI, LNnT, is safe for infants (up to one year of age) when added to infant and follow-on formulae, in combination with 2'-FL, at concentrations up to 0.6 g/L of LNnT and up to 1.2 g/L of 2'-FL, at a ratio of 1:2 in the reconstituted formulae; is safe for young children (older than one year of age) when added to follow-on and young-child formulae, at concentrations up to 0.6 g/L of LNnT (alone or in combination with 2'-FL, at concentrations up to 1.2 g/L, at a ratio of 1:2). The Panel also concludes that LNnT is safe when added to other foods at the uses and use levels proposed by the applicant.

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1. Introduction

1.1. Background and Terms of Reference as provided by the European Commission

On 15 January 2014, the company Glycom A/S submitted a request in accordance with Article 4 of Novel Food Regulation (EC) No 258/97[1] to place on the market lacto-*N*-neotetraose (LNnT) as a novel food ingredient.

On 10 June 2014, the competent authority of Ireland forwarded to the Commission its initial assessment report, which came to the conclusion that LNnT meets the criteria for acceptance of a novel food defined in Article (3)1 of Regulation (EC) No 258/97.

On 7 July 2014, the Commission forwarded the initial assessment report to the other Member States. Several Member States submitted comments or raised objections.

In consequence, a decision is now required by the Commission under Article 7(1) of Regulation (EC) No 258/97.

The concerns of a scientific nature raised by the Member States can be summarised as follows:

- The product specifications for LNnT allows for a lower level of purity (≥ 95 %) compared to the product that was used for actual experiments (98.9 %). The value for this parameter should be adjusted in the product specifications.
- It would be appropriate to include a value for melting point as part of the product specifications. This parameter would provide an indication of both the product purity and identity, particularly given that the applicant relies on HPLC (high-performance liquid chromatography) as a method to assess purity rather than NMR (nuclear magnetic resonance) or elemental analysis.
- Confirmation that the solvents used are acceptable for food use.
- Clarification was requested with respect to the presence of toluene in the novel food ingredient, in particular whether it would be produced by the manufacturing process or whether it would result from the raw material. Concern was expressed with respect to the presence of this compound in the novel food ingredient, in view of its use in infant and follow-on formulae.
- Concern was expressed about possible carry-over of palladium from residual catalyst used in the production process, which may not be removed by filtration. An appropriate maximum limit for palladium could be included in the specifications, especially as the ingredient is intended to be used in infant formulae.
- Clarification was requested on the specification for benzyl-L-LNnT due to discrepancy in values reported in the certificate of analysis and in the specification.
- Clarification was requested on the accreditation of the laboratories which carried out the analyses.
- The dossier only describes in detail the synthesis of LNnT from benzyl-LNnT (the final steps in the production process). This may allow the future use of benzyl-LNnT, produced via a different process. In that event, the applicant will have to reconsider if the specification for benzyl-LNnT is still adequate, in view of the new production process for that compound.
- The estimated intake for distinct population groups, based on food consumption data from the United Kingdom, ranges from 26 to 329 mg/kg body weight per day for the 95th percentile. However, these intake estimates do not include the potential additional intake of LNnT from food supplements, as proposed in the application.
- The intake of large quantities of indigestible carbohydrates can have laxative effects. Since there are not yet any human studies on LNnT, other than those with breast-fed infants, no statement can be made on what levels of LNnT intake could potentially lead to laxative effects.

[1] Regulation (EC) No 258/97 of the European Parliament and of the Council of 27 January 1997 concerning novel foods and novel food ingredients. OJ L 43, 14.2.1997, p. 1–6.

- The potential for genotoxicity was only assessed in two *in vitro* tests for mutagenicity. A test for potential clastogenic activity is lacking (*in vitro* micronucleus test or *in vitro* chromosome aberration test). The absence of genotoxicity for this LNnT-preparation would only be demonstrated sufficiently, upon obtaining a favourable outcome in such study.

- Purified LNnT was assessed in one human study only, which used an LNnT-preparation of unknown purity, produced using an entirely different process. Furthermore, the original study data were not available for an assessment.

- No study attesting to the safety of this oligosaccharide for children under the age of six months, which is a target population for the use of infant formulae, was provided. The Panel on Dietetic Products, Nutrition and Allergies (NDA) of the European Food Safety Authority (EFSA) (2014) recommends that 'the safety of any other non-digestible oligosaccharide, i.e. other than ≤ 0.8 g/100 mL as a mixture of 90 % galacto-oligosaccharides (GOS) and 10 % high molecular weight fructo-oligosaccharides (FOS), or any other new mixture of non-digestible oligosaccharides in infant and follow-on formulae, must be established by clinical evaluation'. It is also recommended that appropriate human studies be carried out to assess the safety of non-digestible oligosaccharides.

In accordance with Article 29(1)(a) of Regulation (EC) No 178/2002,[2] the European Food Safety Authority is asked to carry out the additional assessment for lacto-*N*-neotetraose (LNnT) as a novel food ingredient in the context of Regulation (EC) No 258/97.

EFSA is asked to carry out the additional assessment and to consider the elements of a scientific nature in the comments raised by the other Member States.

2. Data and Methodologies

2.1. Data

The assessment of the safety of this NFI is based on data supplied in the original application, the initial assessment by the competent authority of Ireland, the concerns and objections of the other Member States and the responses of the applicant (see 'Documentation provided to EFSA').

In accordance with Commission Recommendation 97/618/EC,[3] lacto-*N*-neotetraose (LNnT) is allocated to Class 1.2, i.e. 'pure chemicals or simple mixtures from non-genetically modified sources; the source of the NF has no history of food use in the Community'. The data are required to comply with the information required for novel foods of Class 1.2, i.e. structured schemes I, II, IX, X, XI, XII and XIII of Commission Recommendation 97/618/EC. In the current opinion, these structured schemes are listed in sections 3.1 to 3.8. The applicant's intention is to use the NFI in infant formula (IF), follow-on formula (FOF), foods for special medical purposes for infants and young children, and other foods for infants and young children. The applicant also proposed the use of this NFI in foods or food supplements for adults. This assessment concerns only risk that might be associated with consumption, and is not an assessment of the efficacy of LNnT with regard to any claimed benefit.

2.2. Methodologies

The assessment follows the methodology set out in Commission Recommendation 97/618/EC of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and NFIs and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council.

[2] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. OJ L 31, 1.2.2002, p. 1–24.

[3] 97/618/EC: Commission Recommendation of 29 July 1997 concerning the scientific aspects and the presentation of information necessary to support applications for the placing on the market of novel foods and novel food ingredients and the preparation of initial assessment reports under Regulation (EC) No 258/97 of the European Parliament and of the Council. OJ L 253, 16.9.1997, p. 1–36.

3. Assessment

3.1. Specification of the Novel Food Ingredient (NFI)

The NFI is lacto-*N*-neotetraose (LNnT), a synthetic oligosaccharide produced using D-lactose as a starting raw material (Section 3.2).

LNnT is a linear tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose and D-glucose (chemical formula: $C_{26}H_{45}NO_{21}$; molecular weight: 707.63 Da; CAS No 13007-32-4) (Figure 1). The structure can be described as consisting of the two disaccharides N-acetyl-D-lactosamine and D-lactose, which are linked by a β-(1→3) bond.

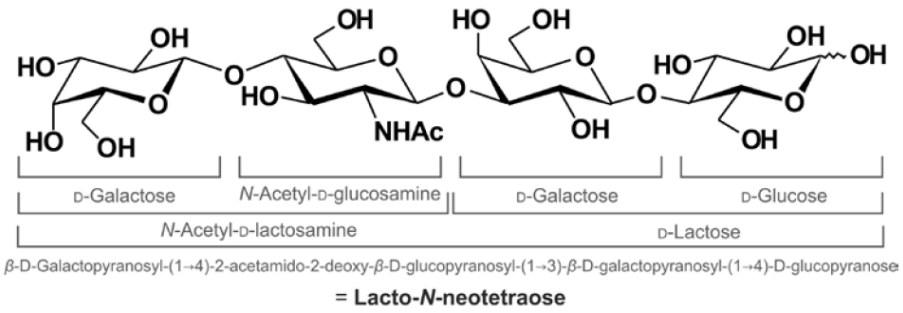

Figure 1: Molecular structure of LNnT

The IUPAC name of LNnT is β- D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose.

LNnT has been characterised by spectroscopic techniques (e.g. by ^{1}H-, ^{13}C-, and 2D-NMR), mass spectrometry (MS) and HPLC. These data are in agreement with the proposed structure (Strecker et al., 1989; Chai et al., 2001). The applicant demonstrated that the NFI is identical to LNnT that is present in human breast milk by comparison with an authentic specimen.

The product specifications for NFI, as proposed by the applicant, are presented in Table 1.

Analytical results from five commercial batches of the NFI, which complied with the product specifications, are presented in Table 2.

The NFI is a white to off-white amorphous powder. In response to a comment from a Member State on the level of purity of LNnT, the applicant indicated that the accuracy of the test method is limited by the hygroscopicity of the ingredient, which binds water upon extended storage and/or sample preparation (uncertainty of the assay is ± 3 %). In response to this comment, the applicant increased the specification of LNnT from 95 % to 96.0 % (HPLC analysis). Results of the analyses of five commercial batches showed that the actual purity of LNnT (water free) was above 98 % (Table 2).

In response to a comment from a Member State, the applicant indicated that, owing to the hygroscopicity of the NFI, the purity and identity of the product cannot be verified by the melting point, which depends on the water content.

Upon a request by EFSA for clarification of the impurities of the NFI, the applicant proposed to include additional specifications for the residual starting material (D-lactose) and two carbohydrate-type side-products, lacto-*N*-triose II and LNnT-fructose isomer, which derive from the manufacturing process. Lacto-*N*-triose II is naturally present in human milk, while the LNnT-fructose isomer is derived from the isomerisation of the terminal glucose moiety of LNnT to fructose. According to the applicant, the proposed specifications for these LNnT-derived impurities have been set to ensure the quality and homogeneity of the NFI and are below any safety-relevant levels.

Table 1: Specifications for LNnT as proposed by the applicant

Parameter	Specification	Method
Appearance	Powder	MSZ ISO 6658:2007
Colour	White to off-white	MSZ ISO 6658:2007
Identification	RT of standard ± 3 %	Glycom method HPLC-102-1C6-003
LNnT (water free)	Min. 96.0 %	Glycom method HPLC-102-1C6-003
D-Lactose[(a)]	Max. 1.0 w/w %	Glycom method HPAEC-102-002
Lacto-N-triose II[(a)]	Max. 0.3 w/w %	Glycom method HPAEC-102-002
LNnT fructose isomer[(a)]	Max. 0.6 w/w %	Glycom method HPLC-102-1C6-003
pH (20 °C, 5 % solution)	5.0 to 7.0	EP method
Water	Max. 9.0 %	Karl-Fischer (EP 2.5.32)
Ash, sulphated	Max. 0.4 %	EP 2.4.14
Acetic acid[(b)]	Max. 0.3 %	MSZ EN ISO 10304-1:2009
Residual solvents	Max. 50 mg/kg singly Max. 200 mg/kg in combination	EP GC 2.4.24
Residual proteins[(c)]	Max. 0.01 %[(d)]	Bradford protein assay
Palladium	Max. 0.1 mg/kg	ICP-MS by EPA 6020A:2007
Nickel	Max. 3.0 mg/kg	ICP-MS by EPA 6020A:2007
Microbiological specifications		
Salmonella	Absent in 25 g	MSZ-EN-ISO 6579:2006
Aerobic mesophilic total count	Max. 500 CFU/g	MSZ-EN-ISO 4833:2003
Enterobacteriaceae	Absent in 10 g	MSZ-ISO 21528-2:2007
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	MSZ-EN-ISO 11290-1:1996, 1998/A1:2005
Bacillus cereus	Max. 50 CFU/g	MSZ-EN-ISO 7932:2005
Yeasts	Max. 10 CFU/g	MSZ-ISO 7954:1999
Moulds	Max. 10 CFU/g	MSZ-ISO 7954:1999
Residual endotoxins[(c,d)]	Max. 10 EU/mg[(d)]	EP 2.6.14

CFU: colony forming units; EP: European Pharmacopoeia; EPA: Environmental Protection Agency; EU: endotoxin units; GC: gas chromatography; HPLC: high-performance liquid chromatography; HPAEC: high-performance anion exchange chromatography; ICP-MS: inductively coupled plasma mass spectrometry; max.: maximum; min.: minimum; RT: retention time.

(a): These specifications refer to the water-free compound.

(b): As free acid and/or sodium acetate.

(c): Specifications for residual proteins and endotoxins are included to allow control on any impurity stemming from potential raw materials used in alternative production processes (i.e. fermentation) to provide reassurance that LNnT produced through Glycom's method of manufacture is controlled for residual levels of proteins and endotoxins.

(d): Specification may be amended (i.e. lowered) should additional data become available in the future.

Table 2: Analyses of five batches of the NFI

Parameter	Specification	Batch results				
		L01032K	**PSD420130 813/158**	**PSD420130 813/159**	**PSD420130 814/160**	**PSD42013 0814/161**
Appearance	Powder	Powder	Powder	Powder	Powder	Powder
Colour	White to off-white	White	White	White	White	White
Identification	RT of standard ± 3%	Complies	Complies	Complies	Complies	Complies
LNnT (water free) (%)	Min. 96 %	98.9	98.7	100.5	100.8	99.8
D-Lactose (%)[(a)]	Max. 1.0 w/w %	<0.03 nd	<0.03 nd	<0.03 nd	<0.03 nd	<0.03 nd
Lacto-*N*-triose II (%)[(a)]	Max. 0.3 w/w %	<0.03 nd	< 0.1	< 0.1	< 0.1	< 0.1
LNnT-fructose isomer (%)[(a)]	Max. 0.6 w/w %	<0.03 nd	0.32	< 0.1	0.56	0.52
pH (20 °C, 5 % solution)	5.0 to 7.0	5.8	6.2	6.0	6.4	6.4
Water (%)	Max. 9.0	1.4	4.0	3.7	3.6	3.4
Ash, sulphated (%)	Max. 0.4	0.2	0.3	0.2	0.3	0.3
Acetic acid[(b)] (%)	Max. 0.3	< 0.02	0.12	< 0.02	0.10	0.14
Residual solvents Singly (mg/kg) Combination (mg/kg)	 Max. 50 Max. 200	 < 50 < 50	 < 10 < 50	 < 10 < 50	 < 10 < 50	 < 10 < 50
Residual proteins (%)[(c)]	Max. 0.01[(c)]	< LOQ[(d)]	< LOQ[(d)]	< LOQ[(d)]	< LOQ[(d)]	< LOQ[(d)]
Palladium (mg/kg)	Max. 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Nickel (mg/kg)	Max. 3.0	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Microbiological specifications						
Salmonella	Absent in 25 g	Complies	Complies	Complies	Complies	Complies
Aerobic mesophilic total count (CFU/g)	Max. 500	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae	Absent in 10 g	Complies	Complies	Complies	Complies	Complies
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Complies	Complies	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25 g	n/a	Complies	Complies	Complies	Complies
Bacillus cereus	Max. 50 CFU/g	n/a	< 10	< 10	< 10	< 10
Yeasts	Max. 10 CFU/g	n/a	< 10	< 10	< 10	< 10
Moulds	Max. 10 CFU/g	n/a	< 10	< 10	< 10	< 10
Residual endotoxins[(c)]	Max. 10 EU/mg [(c)]	Complies	Complies	Complies	Complies	Complies

CFU: colony forming units; EU: endotoxin units; LOQ: limit of quantitation; max.: maximum; min.: minimum; n/a: not applicable; nd: not detected; RT: retention time.
(a): These specifications refer to the water-free compound.
(b): In the form of free acetic acid and/or sodium acetate.
(c): Specification may be amended (i.e. lowered) should additional data become available in the future.
(d): LOQ = 0.0018 w/w %.

The specifications for residual solvents are provided for single and combined solvents, with maximum limits of 50 mg/kg and 200 mg/kg, respectively. In response to a comment from a Member State, the applicant confirmed that all solvents used in the process are used as processing aids. In particular, in response to a comment on toluene, the applicant clarified that toluene, which is not used as a solvent during production, is formed as a volatile product of the hydrogenation reaction of benzyl-LNnT and is removed during the spray drying step (Section 3.2). The applicant also indicated that toluene is controlled by additional internal specifications. Taking into consideration Joint FAO/WHO Expert Committee on Food Additives (JECFA) and European Medicines Agency (EMA) guidance for pharmaceuticals on residual solvents (ICH, 2011; EMA, 2014), the Panel considers that residual solvents do not raise safety concerns.

The protein content is specified at a maximum level of 0.01 %. Batch analyses indicate that protein content is below the limit of quantification (i.e. 0.0018 w/w %) (Table 2).

In response to a comment from a Member State, the applicant indicated that Palladium residue is maintained at levels below 0.1 mg/kg, as confirmed by the results of the batch analyses (Table 2). Nickel is also included in the product specifications (< 3.0 mg/kg), but at levels much lower than the Permitted Daily Exposure levels recommended by EMA for medicinal products. The potential presence of other metals is controlled by internal specifications.

Microbiological contaminants are also controlled by product specifications.

In addition to the product specifications reported in Table 1, the applicant provided further internal product specifications, which have been claimed confidential. The internal product specifications include maximum limits for raw materials, intermediate products (benzyl-LNnT) and processing aids used in the production process. In response to a comment from a Member State, the applicant indicated the maximum limit for benzyl-LNnT, which is included in the internal specifications.

In response to a comment from a Member State, the applicant indicated that analytical measurements were performed using internationally recognised methods in accredited external laboratories. As no internationally recognised methods exist for the analysis of LNnT and LNnT-derived carbohydrates, methods were developed by the applicant and validated under Good Laboratory Practices (GLP).

The Panel considers that the information provided on the specification and data from batch testing do not raise safety concerns.

3.1.1 Stability of the NFI

A 6-month accelerated stability study (40 °C, 75 % relative humidity) has been performed on one batch of crystalline LNnT. Samples for chemical and microbiological analyses were packed into polyethylene bags (primary packaging material), and polyethylene/aluminium/polyester triple layer foil bags (secondary packaging material). LNnT was analysed by HPLC and water content was determined by Karl Fischer titration.

After six months, no significant change was observed in the assay value for LNnT or in the water content (2.2 % after six months vs. 1.8 % at time 0); *N*-acetyl-lactosamine, the potential degradation product of LNnT, was not detected. No unknown degradation products were observed in the HPLC chromatogram. Microbiological purity was maintained throughout the duration of the study.

A 5-year long-term stability study (25 °C, 60 % relative humidity) on one batch of crystalline LNnT was on-going at time of the submission of this dossier. The applicant provided the analytical results obtained up to 36-month point of this long-term stability study for samples of LNnT that were packaged in the same way as in the previous study. After 36 months of storage, no significant changes were observed in the assay value for LNnT or in the water content (2.3 % after 36 months vs. 1.8 % at time 0). After 18 months of storage, neither *N*-acetyl-lactosamine nor unknown degradation products were detected in the HPLC chromatogram (not measured at 24 and 36 months). Microbiological purity was maintained up to 36 months of storage.

Based on the available data, the shelf-life of crystalline LNnT, as proposed by the applicant, is 36 months when stored at room temperature protected from humidity.

The Panel notes that the stability experiments have been performed on a 'crystalline' product which contains 1.8 % to 2.3 % water, whereas the NFI is a 'powder' with a water content of up to 9 %.

However, despite this limitation, the Panel considers that, if protected by adequate packaging, the NFI is sufficiently stable.

3.1.2 Stability under the intended conditions of use

The NFI is intended to be used as an ingredient in IF, FOF, baby foods and a range of food products (Section 3.3). The applicant assessed the stability of the NFI added to IF (stored for three years at 4, 20, 30 and 37 °C), yoghurt (stored for 21 days at 4 °C), citrus juice (stored for 28 days at 4 °C) and ready-to-drink flavoured milk (stored for 14 days (pasteurised) or 28 days (ultra-high temperature (UHT)-treated) at 4 °C). LNnT was added during the production process of these foods, to assess the effects of these processing on the NFI, as well as the stability of the NFI in storage. The content of LNnT was measured by HPLC at different time-points over the course of the studies. No significant losses of LNnT were observed in any of the conditions tested which included pasteurisation and UHT treatments.

The Panel considers that the data provide sufficient information with respect to the stability of the NFI.

3.2. Effect of the production process applied to the NFI

LNnT is produced by chemical synthesis through a two-stage manufacturing process.

In the first stage, the 'chemically protected' LNnT tetrasaccharide is produced by a selective β-(1→3)-glycosylation reaction between two key intermediates, the 'chemically protected' and activated *N*-acetyl-D-lactosamine and the selectively protected D-lactose, both obtained from D-lactose by two parallel processes. The 'protected' LNnT tetrasaccharide is then converted into the intermediate benzyl-LNnT and recrystallised. The applicant provided internal specifications for the benzyl-LNnT raw material. In response to a comment from a Member State, the applicant described the synthesis of benzyl-LNnT in details (confidential).

In the second stage, benzyl-LNnT is hydrogenated in the presence of catalytic amounts of Pd/C, to obtain the LNnT product, which undergoes a series of steps, including purification and spray drying.

LNnT is manufactured in compliance with good manufacturing practice procedures. The applicant indicates that specifications are set according to hazards analysis and critical control points principles, as defined by Regulation (EC) No 852/2004,[4] and conformity is monitored by certificates of analysis at each key stage of the production process.

The Panel concludes that the production process does not cause safety concerns.

3.3. Anticipated intake/extent of use of the NFI

3.3.1 Uses for infants and young children

The applicant intends to market LNnT as an ingredient in IF and FOF, including formulae for special medical purposes, and other foods for infants and young children (Table 3). The proposed maximum use levels of LNnT in all other food categories are based on the applicant's consideration of providing a similar intake as to that of LNnT from breast milk on a mg/kg body weight basis.

[4] Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. OJ L 139, 30.4.2001, p. 1–54.

Table 3: Food uses for LNnT for infants and young children proposed by the applicant

Food category name	Proposed maximum use level
Foods for infants and young children	
Infant formulae as defined by Commission Directive 2006/141/EC[5]	0.6 g/L of reconstituted formula
Follow-on formulae as defined by Directive 2006/141/EC[5]	0.6 g/L of reconstituted formula
Processed cereal-based foods and baby foods for infants and young children as defined by Commission Directive 2006/125/EC[6]	0.6 g/100 g for solid foods
Other foods for young children	0.6 g/100 g for solid foods 0.6 g/L for beverages as consumed
Dietary foods for infants and young children for special medical purposes as defined by Commission Directive 1999/21/EC[7] and special formulae for infants	As specified on a case-by-case basis in accordance with Commission Directive 1999/21/EC

For infants aged 0 to 6 months, the estimated intake of LNnT was calculated from the use of LNnT in IF alone, under the assumption that it would be the only source of LNnT in this population group. The daily intake of liquid IF was estimated to be 1,060 mL/day for infants aged 0 to 6 months, based on the food consumption data used by EFSA (EFSA AFC Panel, 2006). This food intake scenario was based on a 3-month-old infant weighing 6.1 kg consuming 174 mL/kg body weight per day of IF at the 95th percentile. Assuming an intended use level of 0.6 g LNnT/L formula, the 95th percentile daily intake of LNnT from its use in IF alone is estimated to be 637 mg/day (104 mg/kg body weight per day) in infants aged 0 to 6 months.

For infants aged 4 to 17 months, the applicant estimated intakes of LNnT from the consumption of IF and other infant-specific foods and beverages based on data available from the UK Diet and Nutrition Survey on Infants and Young Children (DNSIYC) conducted in 2011 (NatCen Social Research et al., 2013). Food consumption was estimated through a 4-day dietary diary. Intake estimates of LNnT were calculated based on the proposed use levels expressed in Table 3 for the category 'Foods for Infants and Young children'. The percentage of users of foods from this category was high among all age groups (> 84.7 %), with the highest percentage of users found among infants aged 4 to 6 months (98.1 %).

It was determined that infants aged 4 to 6 months have the highest mean all-user intake of LNnT on an absolute basis of 1.11 g per day, while infants aged 7 to 12 months had the highest 95th percentile all-user intakes (2.77 g per day) (Table 4). Young children aged 13 to 17 months had the lowest mean and 95th percentile all-user intakes of 0.54 and 1.73 g/day, respectively.

On a body weight basis, infants aged 4 to 6 months were identified as having the highest mean and 95th percentile all-user intakes of any population group of 135 and 329 mg/kg body weight per day, respectively (Table 5). Young children aged 13 to 17 months had the lowest mean and 95th percentile all-user intakes of 50 and 159 mg/kg body weight per day, respectively (Table 5).

[5] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC. OJ L 401, 30.12.2006, p. 1–33.
[6] Commission Directive 2006/125/EC of 5 December 2006 on processed cereal-based foods and baby foods for infants and young children. OJ L 339, 6.12.2006, p. 16–35.
[7] Commission Directive 1999/21/EC of 25 March 1999 on dietary foods for special medical purposes. OJ L 91, 7.4.1999, p. 29-36.

Table 4: Summary of the estimated daily intake of LNnT by infants and young children in the UK, per person, based on data from DNSIYC 2011

Population group	Age group (months)	Total (n)	All-person consumption[(a)] (g/day)		All-user consumption[(a)] (g/day)			
			Mean	95th percentile	%	n	Mean	95th percentile
Infants	4–6	329	1.08	2.69	98.1	323	1.11	2.75
Infants	7–12	1 319	1.01	2.75	94.8	1 252	1.07	2.77
Young children	13–17	1 035	0.37	1.54	67.5	688	0.54	1.73
Total	4–17	2 683	0.77	2.37	84.7	2 263	0.91	2.55

(a): For the DNSIYC, only intakes based on the category 'Foods for infants and young children' were included in the assessment

Table 5: Summary of the estimated daily intake of LNnT by infants and young children in the UK, per kg body weight, based on data from DNSIYC 2011

Population group	Age group (months)	Total (n)	All-person consumption[(a)] (mg/kg body weight per day)		All-user consumption[(a)] (mg/kg body weight per day)			
			Mean	95th percentile	%	n	Mean	95th percentile
Infants	4–6	329	133	328	98.1	323	135	329
Infants	7–12	1 319	107	293	94.8	1 252	113	295
Young children	13–17	1 035	34	142	67.5	688	50	159
Total	4–17	2 683	82	260	84.7	2 263	97	276

(a): For the DNSIYC, only intakes based on the category 'Foods for infants and young children' were included in the assessment

3.3.2 Other food uses for other population groups

The applicant intends to market LNnT as an ingredient in a variety of food products including food supplements (Table 6). In a tiered approach the applicant first estimated intakes of the NFI by using the EFSA Food Additive Intake Model (FAIM) tool which is based on summary statistics of consumption data of the EFSA Comprehensive Food Consumption Database (EFSA, 2012). For a refined intake estimate, the applicant used individual consumption data recorded by the UK National Diet and Nutrition Survey (NDNS) for the years 2008–2010 (UKDA, 2012).

The proposed maximum use levels of LNnT in all food categories are based on the applicant's consideration for providing similar intakes as that of LNnT from breast milk in infants on a mg/kg body weight basis.

Table 6: Uses and use Levels for LNnT (g/kg)[(a)] proposed by the applicant

UK NDNS food categories	Food classification system used by the FAIM Tool	Food category name	Suggested serving size	Maximum level per serving	Proposed maximum use level (g/kg)
Dairy products and analogues:	1.1	Unflavoured pasteurised and sterilised (including UHT) milk	200 g	0.6 g/L	0.6
Unflavoured pasteurised, sterilised milk and milk–recipes,	1.2	Unflavoured fermented milk products	125 g	0.6 g/L for beverages. Other foods: 0.6–1.2 g/serving	4.8–9.6
	1.3	Unflavoured fermented milk products, heat-treated after fermentation			4.8–9.6
Unflavoured fermented milk products,	1.4	Flavoured fermented milk products including heat-treated products			4.8–9.6
Flavoured fermented milk products,	1.8	Dairy analogues, including beverage whiteners	200 g	0.6 g/L for beverages. Other foods: 0.6–1.2 g /serving	0.6–6
Dairy analogues.			3 g	0.6 g per sachet whitener	200
Cereal bars	7.2	Fine bakery wares. Cereal bars only	n/a[(b)]	0.6 g/100g	6
Table top sweeteners	11.4	Table top sweeteners	6 g sachet	0.6 g per sachet	100
Infant formula and follow-on formula,	13.1.1[(d)]	Infant formulae as defined by Commission Directive 2006/141/EC[8]	n/a[(c)]	0.6 g/L of reconstituted formula	0.6
	13.1.2[(d)]	Follow-on formulae as defined by Directive 2006/141/EC	n/a[(c)]	0.6 g/L of reconstituted formula	0.6
Foods for infants and young children,	13.1.3[(d)]	Processed cereal-based foods and baby foods for infants and young children as defined by Commission Directive 2006/125/EC[9]	n/a[(b)]	0.6 g/100g for solid foods	6
Dietary foods (including powders) for weight control diets,	13.1.4[(d)]	Other foods for young children	n/a[(b)]	0.6 g/100g for solid foods	6
				0.6 g/L for beverages as consumed	0.6
Foods suitable for people intolerant to gluten.	13.2	Dietary foods for special medical purposes defined in Directive 1999/21/EC[10] (excluding products from food category 13.1.5) [(e)]	Case-by-case basis	As specified on a case-by-case basis in accordance with	Case-by-case basis

[8] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC. OJ L 401, 30.12.2006, p. 1-33.
[9] Commission Directive 2006/125/EC of 5 December 2006 on processed cereal-based foods and baby foods for infants and young children. OJ L 339, 6.12.2006, p. 16-35.

UK NDNS food categories	Food classification system used by the FAIM Tool	Food category name	Suggested serving size	Maximum level per serving	Proposed maximum use level (g/kg)
				Commission Directive 1999/21	
	13.3	Dietary foods for weight control diets intended to replace total daily food intake or an individual meal (the whole or part of the total daily diet)	250 g drinks, 30 g bars	0.6 g/meal replacement	2.4 (drinks), 20 (bars)
	13.4	Foods suitable for people intolerant to gluten as defined by Commission Regulation (EC) No 41/2009[11]	Bread products 40 g, pastas 55 (unprepared) to 110 g (prepared)	0.6–1.2 g /serving	5.5–30
Beverages: Fruit & vegetable juices and nectars, Flavoured drinks, Coffee, tea (except black tea), Herbal and fruit infusions.	14.1.2	Fruit juices as defined by Directive 2001/112/EC and vegetable juices[12]	n/a[(b)]	0.6 g/L	0.6
	14.1.3	Fruit nectars as defined by Directive 2001/112/EC and vegetable nectars and similar products	n/a[(b)]	0.6 g/L	0.6
	14.1.4	Flavoured drinks	n/a[(b)]	0.6 g/L	0.6
	14.1.5	Coffee, tea (excluding black tea), herbal and fruit infusions, chicory; herbal and fruit infusions and chicory extracts; tea, plant, fruit and cereal preparations for infusions, as well as mixes and instant mixes of these products	250	0.6–1.2 g /serving	2.4–4.8
Food supplements as defined in Directive 2002/46/EC[13]	17.1	Food supplements supplied in a solid form including capsules and tablets and similar forms, excluding chewable forms	n/a[(b)]	1.5 g per day	Maximum intake of 1.5 g/day[(f)]
	17.2	Food supplements supplied in a liquid form	n/a[(b)]		
	17.3	Food supplements supplied in a syrup-type or chewable form	n/a[(b)]		

EC: European Commission; EU: European Union; n/a, not applicable.
(a): While it is proposed that LNnT will be added to these food categories, including 'Dairy products and analogues', once placed on the market, all food products with added LNnT would be appropriately reclassified and labelled.

[10] Commission Directive 1999/21/EC of 25 March 1999 on dietary foods for special medical purposes. OJ L 91, 7.4.1999, p. 29–36.
[11] Commission Regulation (EC) No 41/2009 of 20 January 2009 concerning the composition and labelling of foodstuffs suitable for people intolerant to gluten. OJ L 16, 21.1.2009, p. 3–5.
[12] Council Directive 2001/112/EC of 20 December 2001 relating to fruit juices and certain similar products intended for human consumption. OJ L 10, 12.1.2003, p. 58–66.
[13] 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 dietary supplements. OJ L 183, 12.7.2002, p. 51–57.

(b): A serving size is not provided on the basis that the use level is specified on a per L/kg basis and not on a per serving basis.
(c): The suggested serving size for infant formula is according to the manufacturer's instructions and according to the baby's age and weight.
(d): Food Classification System (FCS, Categories 13.1.1, 13.1.2, 13.1.3 and 13.1.4 were also assessed using food consumption data from the DNSIYC).
(e): Category 13.2 was not included in the UK NDNS intake assessment as these products should be assessed on a case-by-case basis, and are not widely consumed by the general population.
(f): Food Supplements were not considered in the combined intake assessment as only a maximum of 1.5 g of LNnT per day through a single supplement dose would be recommended

Mean and high-intake estimates derived with the EFSA FAIM tool are presented in Table 7. A total of 26 different dietary surveys carried out in 17 different European countries are included in the FAIM tool. Mean intake values for the total population are calculated per age group by summing up the mean exposures from all contributing food sources in a given survey. High-level values of a given food category are calculated either as the 95th percentile of consumers-only, when the number of consumers is ≥ 60, or as the mean of consumers only, when the number of consumers is < 60.

The applicant used the lower and upper ends of the proposed use level ranges per food category for the calculation in the FAIM tool. The food categories included in the calculations were dairy products and analogues, bakery wares, sugars, syrups, honey and table-top sweeteners, foods intended for particular nutritional uses as defined by Directive 2009/39/EC[14] and non-alcoholic beverages, as per the proposed uses. The results are presented in Table 7. The mean intakes of LNnT in the total population, as reported in the FAIM tool, ranged from 15 to 48 mg/kg body weight per day in adolescents and up to 120 to 212 mg/kg body weight per day in toddlers when the maximum use levels were used (Table 7). High-level intakes ranged from 34 to 118 mg/kg body weight per day in adolescents and up to 370 to 528 mg/kg body weight per day in toddlers when the maximum use levels were used.

The Panel notes that the EFSA FAIM tool has been developed as a screening tool for deriving chronic intake estimates for food additives for different population groups throughout several European countries. The tool is based on summary statistics of the EFSA Comprehensive database. The FoodEx classification used by this tool has been linked mainly to the level 2, and for few foods up to level 3, of the Food Classification System (FCS) as presented in Commission Regulation (EU) N° 1129/2011[15]. Intake levels from all contributing food sources are obtained by adding the high-level (usually the 95th percentile) of exposure from the food category providing the highest estimate plus the estimated mean exposure values for the remaining categories. The underlying methodology of the FAIM tool generally leads to an overestimation of the estimated intakes as expressed by the EFSA instructions for the use of the FAIM tool (EFSA, 2012).

Table 7: Summary of estimated intakes of LNnT using the EFSA FAIM tool

Population group	Age	Mean intakes (mg/kg body weight per day)		High level intakes (mg/kg body weight per day)	
		Lower use level	Maximum use level	Lower use level	Maximum use level
Toddlers	12 to 35 months	41–101	120–212	99–225	370–528
Children	3 to 9 years	26–76	36–129	52–181	86–338
Adolescents	10 to 17 years	12–30	15–48	22–65	34–118
Adults	18 to 64 years	11–43	18–76	25–81	45–152
Elderly	65 years and older	9–41	15–79	18–74	45–143

For the refined intake estimate based on UK individual consumption data, individual food codes representative of each proposed food use were taken from the food code list associated with the UK NDNS food consumption survey and grouped according to the proposed uses for the LNnT assessment presented in Table 6. The daily intake of LNnT was calculated at individual level, and represents projected 4-day averages for each individual from days 1 to 4 of NDNS data, in the UK population. High percentile intake estimates were derived from the distribution of these average amounts. Results are provided in Tables 8 and 9.

In relation to all-user intakes, toddlers had the highest mean intakes of about 0.9 g per day. Among adults, women of child-bearing age had the highest intakes at the 95th percentile at 3.3 g per day (Table 8). On a body weight basis, toddlers were identified as having the highest mean and 95th percentile intake of any population group of 63 and 132 mg/kg body weight per day, respectively (Table 9).

[14] European Parliament and the Council of the European Union. Directive 2009/39/EC of the European Parliament and of the Council of 6 May 2009 on foodstuffs intended for particular nutritional uses. OJ L 124, 20.05.2009, p. 21–29.

[15] Commission Regulation (EU) No 1129/2011 of 11 November 2011 amending Annex II to Regulation (EC) No 1333/2008 of the European Parliament and of the Council by establishing a Union list of food additives. OJ L 295, 11.11.2011, p.1–177.

Table 8: Summary of the estimated daily intake of LNnT from proposed food categories by different population groups in the UK, per person, based on data from NDNS

Population group	Age group (years)	All-person consumption (g/day)		All-user consumption (g/day)			
		Mean	95th percentile	%	n	Mean	95th percentile
Toddlers	1–3	0.89	1.90	100.0	219	0.89	1.89
Children	4–10	0.78	1.74	100.0	423	0.78	1.74
Teenagers	11–18	0.73	1.93	99.8	451	0.74	2.93
Women of child-bearing age	19–40	0.86	3.32	100.0	216	0.86	3.32
Female adults	19–64	0.85	2.51	99.9	460	0.85	2.51
Male adults	19–64	0.71	2.15	99.6	344	0.72	2.15
Elderly adults	65 years and older	0.68	1.80	99.8	223	0.68	1.80

Table 9: Summary of the estimated daily per kilogram body weight intake of LNnT from proposed food categories by different population groups in the UK, based on data from NDNS

Population group	age group (years)	All-person consumption (mg/kg body weight per day)		All-user consumption (mg/kg body weight per day)			
		Mean	95th percentile	%	n	Mean	95th percentile
Toddlers	1–3	63	132	100.0	219	63	132
Children	4–10	31	72	100.0	423	31	72
Teenagers	11–18	13	35	99.8	451	13	35
Women of child-bearing age	19–40	13	53	100.0	216	13	53
Female adults	19–64	13	40	99.9	460	13	40
Male adults	19–64	9	26	99.6	344	9	26
Elderly adults	65 years and older	9	28	99.8	223	9	28

One Member State noted that the intake estimates calculated by the applicant do not include the potential additional intake of LNnT from food supplements, as proposed in the application. According to the applicant, the most significant target group for food supplements will be women of child-bearing age. Considering the highest estimated 95th percentile daily intake of LNnT at 3.3 g for women of child-bearing age plus the maximum intended daily intake from food supplements of 1.5 g, LNnT intakes in this population group could reach up to 4.8 g per day.

The Panel considers that the refined intake estimate based on UK individual consumption data is sufficiently conservative, as it is based on the assumption that all proposed food items consumed by an individual actually contain the NFI at the maximum specified level of use.

3.4. Information from previous exposure to the NFI or its source

LNnT is a naturally occurring tetrasaccharide that is present in human milk (i.e. human milk oligosaccharides (HMO)) (Urashima et al., 2011a, b). Concentrations of LNnT are highest in colostrum (up to 550 mg/L at days 3 to 10 post-partum) and decrease thereafter (200 mg/L at days 31 to 452 post-partum) (Erney et al., 2000).

The applicant provided several publications on the LNnT content in human milk in relation to secretor and Lewis-blood group status (Thurl et al., 1997, 2010; Galeotti et al., 2012), ethnicity (Erney et al., 2000), lactation period (Coppa et al., 1999; Erney et al., 2000; Sumiyoshi et al., 2003; Asakuma et al., 2008; Leo et al., 2010; Bao et al., 2013), preterm birth (Nakhla et al., 1999; Gabrielli et al., 2011) and in mature human milk (Chaturvedi et al., 1997, 2001; Asakuma et al., 2011).

According to the applicant, the content of LNnT in mature breast milk ranged from 110 to 630 mg/L; however concentrations up to 1,390 and 2,500 mg/L have also been reported (Coppa et al., 1999; Galeotti et al., 2012). Based on the content of LNnT in mature breast milk and on the breast milk consumed by a 6.5 kg infant (around 1 L per day) (Davies et al., 1994; Hester et al., 2012), the applicant estimated that the intake of natural LNnT from breast milk would be approximately 20 to 100 mg/kg body weight per day and potentially up to 385 mg/kg body weight per day.

The Panel notes that the history of human exposure to LNnT is limited primarily to that of breast-fed infants. The Panel also notes that the proposed maximum intake level of the NFI by infants is within the range of intake levels from breast milk reported in the literature.

3.5. Nutritional information on the NFI

The applicant proposed the addition of LNnT to IF, in order to bring IF and FOF closer to the composition of breast milk. The applicant also proposed the addition of LNnT to other food categories, such as dairy products and analogues, bakery wares, sugars, syrups, honey and table-top sweeteners, PARNUTS (foodstuffs for particular nutritional uses) and beverages.

The impact of uses other than IF would be comparable to the current use of fibre-type oligosaccharides (e.g. GOS, FOS), which are added to a range of food products.

In response to a comment from a Member State with respect to the potential laxative effect of the NFI, the applicant indicated that proposed uses will not result in a higher consumption than that of breast milk.

The Panel considers that the consumption of the NFI is not nutritionally disadvantageous.

3.6. Microbiological information on the NFI

Microbiological specifications, which also include microbial endotoxins, are presented in Table 1 (section 3.1). The applicant provided analytical results for five batches which were compliant with the microbiological specifications (Table 2, section 3.1).

The Panel considers that the microbiological information provided does not raise safety concerns.

3.7. Toxicological information on the NFI

3.7.1 Absorption, distribution, metabolism and excretion

HMOs, including LNnT, are reported to be resistant to hydrolysis by digestive enzymes in *in vitro* studies (Engfer et al., 2000; Gnoth et al., 2000).

HMO fermentation in the colon in infants who received a load of HMO (a purified oligosaccharide fraction from their mothers' milk) was determined through a breath hydrogen test. The observations from this test suggest that HMOs, consumed as a load, are fermented in the colon (Brand-Miller et al., 1995, 1998).

The pattern and the amount of HMO in the faeces of breast-fed infants were compared with the pattern and amount of HMO in breast-milk (Chaturvedi et al., 2001; Coppa et al., 2001). These studies report that 40–50 % and 97 %, respectively, of the ingested amount of HMO is excreted unchanged in the faeces. Other studies reported the pattern of HMO (including LNnT) in the faeces of breast-fed infants (Albrecht et al., 2010, 2011a, b; De Leoz et al., 2013).

Urinary excretion of HMO has been investigated in breast-fed infants. A bolus doses of ^{13}C-labelled glucose or ^{13}C-labelled galactose was consumed by lactating mothers in order to label the oligosaccharides in their breast milk. ^{13}C-labelled HMO was detected in the urine of their breast-fed infants (Obermeier et al., 1999; Rudloff et al., 2006, 2012; Rudloff and Kunz, 2012; Dotz et al., 2014). Another study compared the amount of oligosaccharides in the urine of breast-fed infants with the oligosaccharides in their mothers' milk (Chaturvedi et al., 2001). These studies suggest that a small proportion of HMO (approximately 1 to 2 % of the ingested amount) is excreted unchanged in infants' urine. Data from *in vitro* studies using the Caco-2 human intestinal epithelial cell model suggest that

neutral HMOs (like LNnT) are transported across the intestinal epithelium by receptor-mediated transcytosis, as well as by paracellular pathways (Gnoth et al., 2001).

Overall, the data indicate that a large proportion of the ingested HMO, including LNnT, reaches the large intestine and is either fermented by the intestinal microbiota or excreted unchanged in the faeces.

There are no indications that the absorption of the NFI may differ from that of LNnT from breast milk, and the consumption levels of the NFI would likely be similar to those of LNnT from breast milk by infants.

The Panel considers that the limited amount of the NFI that could be absorbed, as suggested by the data provided, does not raise safety concerns.

3.7.2 Genotoxicity

The applicant provided three studies on the potential mutagenicity of LNnT (purity of 98.9 %) (Coulet et al., 2013; unpublished study report, 2015a). These studies were conducted in compliance with the Organisation for Economic Co-operation and Development (OECD) principles of GLP and the appropriate OECD test guidelines.

In the bacterial reverse mutation assay (Ames test), five *Salmonella typhimurium* strains (TA98, TA100, TA1535, TA1537 and TA102) were exposed to LNnT, at levels up to 5,000 µg/plate, in the presence or absence of metabolic activation (S9-mix), using the plate-incorporation and pre-incubation methods (OECD test guideline No 471; OECD, 1997a) (Coulet et al., 2013). No signs of cytotoxicity or precipitation were observed for any strain treated with LNnT in the presence or absence of S9-mix. No significant increases in the number of revertants were observed at any concentration of LNnT, compared with the negative control, in either the presence or the absence of S9-mix. This study showed no mutagenicity of LNnT, with or without S9-mix, up to the highest tested concentration of 5 000 µg/plate.

In the *in vitro* mammalian cell gene mutation test, L5178Y $Tk^{+/-}$ mouse lymphoma cells were incubated with LNnT for four hours, with or without S9-mix, and for 24 hours without S9-mix (Coulet et al., 2013). This study was conducted in compliance with OECD test guideline No 476 (OECD, 1997b). No signs of precipitation or cytotoxicity were observed in the cells exposed to any concentration of LNnT. No statistically significant increases in the frequency of mutations were observed in cells treated with LNnT, with or without S9-mix. This study showed no mutagenicity of LNnT, with or without S9-mix, up to the highest tested concentration of 4 250 µg/mL.

In response to comments from some Member States, the applicant provided the study report on an *in vitro* micronucleus assay with LNnT (OECD test guideline No 487; OECD, 2014) (unpublished study report, 2015a). LNnT was tested using human peripheral blood lymphocytes, with or without metabolic activation (S9-mix). The highest concentration tested was 2 000 µg/mL. In the first experiment, LNnT was tested for three hours with a 27-hour harvest time, with or without S9-mix, whereas, in the second experiment, the exposure lasted 24 hours with a 24-hour harvest time, without S9-mix. LNnT did not induce a statistically significant relevant increase in the number of mono- or binucleated cells with micronuclei with or without S9-mix. This study showed neither clastogenicity nor aneugenicity of LNnT, with or without S9-mix, up to the highest tested concentrations of 2 000 µg/mL.

The Panel considers that the information provided does not raise safety concerns as regards the genotoxicity of the NFI.

3.7.3 Repeated exposure toxicity studies

The potential toxicity of the NFI was investigated in a sub-chronic (90-day) oral toxicity study in Wistar rats, which was preceded by a 14-day dose range-finding study and a 28-day repeated-dose study (Coulet et al., 2013). In all studies synthetically produced LNnT with a purity of 98.9 % was used as the test material.

Rat pups were used to adapt the standard OECD test guideline No 408 to the intended use of the NFI in infants (OECD, 1998a; Barrow, 2007). In all studies, FOS was given to a reference control group on the basis that they are used in IF.

In the non-GLP 14-day dose range-finding study with Wistar (Crl:WI(Han)) rat pups, 0 (water vehicle control), 1,000, 2,500, or 5,000 mg LNnT/kg body weight per day were administered, by gavage, from post-natal day (PND) 7 until PND 20 (weaning) (Coulet et al., 2013). No mortality and no abnormalities in clinical signs, body weights, or necropsy were observed in rats administered LNnT. Based on these observations, the authors considered that the highest dose of LNnT suitable for longer-term rat studies was 5,000 mg/kg body weight per day.

In the 28-day repeated-dose study and in the sub-chronic 90-day toxicity study, Wistar (Crl:WI(Han)) rat pups were administered daily the NFI by gavage at doses of 0 (water vehicle control), 1,000, 2 500, or 5 000 mg/kg body weight for 28 or 90 consecutive days, respectively, starting from PND 7 (Coulet et al., 2013). These studies were conducted in compliance with the OECD principles of GLP and in accordance with OECD test guidelines No 407 and No 408 (OECD, 1998a, b, 2008), respectively.

In the 28-day repeated-dose study, haematological tests showed significant increases in total white blood cell (WBC) and absolute monocyte counts in the high-dose LNnT female group compared with the control group, while these changes were not observed in the male groups. Other changes in haematological parameters were observed: a significant decrease in reticulocytes and mean cell volume in the mid- and high-dose LNnT male groups compared with the control group, and a significant increase in mean corpuscular haemoglobin concentration in the high-dose LNnT male group compared with the control group. These changes, which were slight in magnitude and not associated with any clinical or histopathological changes in the spleen, lymph nodes, heart, lungs or liver, were observed in the in the mid- and high-dose LNnT male groups in comparison with the control group. No changes in organ weights or macroscopic findings or histopathological findings were observed.

At day 90 of the sub-chronic study, haematological and serum examinations were performed only in rats from the control, high-dose LNnT and FOS groups, as the animals from the low- and mid-dose LNnT groups were not sampled owing to an oversight in the study plan. Therefore, haematological and serum examinations for all the LNnT groups, including the low- and mid-dose LNnT groups, were performed at day 100 and at the end of the 4-week recovery period (day 119). At day 90, WBC and absolute lymphocyte counts were significantly reduced in the high-dose LNnT males (by 14 % and 10 %, respectively). At days 100 and 119, WBC counts remained significantly lower in males administered LNnT at all doses than in control males. In females, WBC counts were significantly reduced only at day 100 in the high- and mid-dose LNnT groups compared with the control group.

At day 90, a significant decrease in reticulocytes and platelet counts was observed in males in the high-dose LNnT group compared with the control group. No significant changes were observed in reticulocytes at day 100 or 119 in any of the groups. A significant decrease in platelet counts was observed in males in the low-dose LNnT group at day 100.

A significant decrease in haemoglobin (Hb) levels and in packed cell volume (PCV) at day 90 was observed in females, but not males, in the high-dose LNnT group. No significant changes were observed in absolute monocyte counts in the 90-day study, whereas an increase was observed in the high-dose LNnT female group in the 28-day study.

Decreased zymogen content was seen in acinar cells in one female and two males from the high-dose LNnT group. The changes were of minimal to slight severity. At the end of the recovery period, this change, of minimal severity, was seen in one male and one female from the high-dose group.

In both the 28-day repeated-dose study and the sub-chronic 90-day study, few animals in the high-dose LNnT group exhibited soft, liquid, yellow-coloured faeces and erythema on the urogenital area before weaning. Such findings were not observed in the control or low- and mid-dose LNnT groups.

Taking into account the observations as regards WBC counts from the 28-day and 90-day studies, the Panel noted that the significant increase in WBC count in high-dosed LNnT females in the 28-day study was not observed in females in the 90-day study. In contrast, a significant reduction in WBC count was reported in the high- and mid-dose LNnT female groups at day 100, together with significant lower WBC count in males from all LNnT dose groups. The Panel notes the inconsistency of

the observations as regards WBC regarding the sex of the rats across the two studies and notes that no dose-effect relationship was observed. The Panel regards these findings on WBC not of being adverse effects related to the test compound.

Based on the observations on reticulocytes, platelet counts, Hb levels and PCV in the high-dose LNnT group (5 000 mg/kg body weight per day) and the decrease in the zymogen content in acinar cells in three animals in the high-dose LNnT group, the Panel considers that the no observed adverse effect level (NOAEL) is 2 500 mg/kg body weight per day.

3.7.4 Human studies

The applicant provided a double-blind, placebo-controlled study (Prieto, 2005) on oropharyngeal colonisation of *Streptococcus pneumoniae* in infants and young children (aged 6–24 months) who were randomised to consume *ad libitum* a formula containing 200 mg/L of LNnT (n = 115) or the same formula without LNnT (n = 113) for 16 weeks. LNnT was produced through a yeast fermentation process, and it was reported to contain less than 2 % of lactose and no other detectable contaminants. The Panel notes that LNnT that was used in this study was produced with a different manufacturing process from the one proposed for the NFI. The Panel considers that this study is of limited value and not pivotal to the safety assessment of the use of NFI in infants.

One Member State indicated the absence of studies on the safety of the NFI in infants aged 0-6 months. In response to this comment and to a request by EFSA, the applicant provided an unpublished study report on a double-blind, randomised, controlled clinical trial on the effect of a combination of two oligosaccharides, LNnT and 2'-*O*-fucosyllactose (2'-FL), in infants (unpublished study report, 2015b, d). The Panel notes that 2'-FL is subject to another application as a NFI which has been assessed in parallel with the safety assessment of LNnT (EFSA NDA Panel, 2015). A total of 175 healthy, full-term infants (aged 0–14 days) were randomised to consume either a formula with added LNnT and 2'-FL (n = 88; 0.5–0.6 g/L of LNnT and 1.0–1.2 g/L of 2'-FL for reconstituted formula) or the same formula without oligosaccharides (n = 87) up to six months of age. The formula used was a standard whey-predominant starter IF with long-chain polyunsaturated fatty acids and without oligosaccharides (66.9 kcal/100 mL reconstituted formula, 1.9 g protein/100 kcal powder with a whey:casein ratio of 2.5:1 (71.6 % whey and 28.4 % casein)). Exclusively breast-fed (BF) infants (beginning at three months of age) were included as a reference group.

This study aimed to demonstrate non-inferiority in weight gain between the two formula-fed groups up to four months of age (primary outcome). Differences in body weight, body length, head circumference, 'digestive tolerance', stool characteristics, behaviour patterns, formula intake, use of concomitant medications and adverse events between the two formula-fed groups were also investigated (secondary outcomes).

The study showed that the weight gain in the test group was non-inferior to the weight gain in the control group in both the intention-to-treat (ITT) and per-protocol (PP) populations (n = 71 in the test formula group and n = 75 in the control formula group) (in the ITT population, −0.13 g/day (two-sided 95 % confidence interval (CI) −1.63 to 1.37; p = 0.864); in the PP population, −0.30 g/day (two-sided 95 % CI −1.94 to 1.34; p = 0.715)).

Mean weight, length, head circumference and BMI of infants aged 0-4 months were comparable with the corresponding World Health Organization (WHO) standard growth curves. No differences in mean weight, length, head circumference or BMI between groups were observed at any monthly visits (except for lengths at the 3-month visit).

Stool consistency scores on the Bristol Stool scale were not significantly different between the test and control formula-fed groups, except for scores for 2-month-old infants in the test formula group, who had higher scores (i.e. softer stools) than infants who consumed the control formula. No significant difference was observed in the number of stools reported by parents between the two formula-fed groups.

Overall, adverse events were not significantly different between the test and control formula-fed groups, except for the incidence of bronchitis, which was significantly lower in infants in the intervention group than in those in the control group (odds ratio (OR) = 0.30; 95 % CI 0.11–0.73;

p = 0.004). Antibiotic use was significantly lower in infants in the intervention group as compared with those in the control group (25.0 % vs. 41.4 %; OR = 0.47; 95 % CI 0.23–0.94; p = 0.025).

Microbiota composition and the presence of pathogens in stools from infants of three months of age were reported. The alpha diversity of the microbiota of the BF group was significantly lower than the diversity of both formula groups, but the diversity of the test group was significantly reduced compared with the diversity of the control formula group and, therefore, closer to the BF group. An increase of *Bifidobacterium,* a decrease in *Escherichia* and unclassified *Peptostreptococcaceae* were reported for infants in the test formula group compared with the infants in the control formula group, showing proportions closer to the BF group. The number of infants with at least one detectable viral, bacterial or eukaryotic (protista) pathogen in stool samples was not statistically different between the two formula-fed groups.

The Panel notes that this study showed no difference in growth in infants who consumed a formula added with the combination of LNnT and 2'-FL (at the concentrations tested in the study) compared with the control formula infants, and that the growth curves were comparable to the WHO standard curves. The Panel also notes that the results on stool and microbiota endpoints do not raise safety concerns.

The applicant provided an unpublished study report on a placebo-controlled, double-blind, parallel, dose-response trial in 100 healthy adults (49 females, age range: 19–57 years) who were randomised into 10 groups (n = 10) to consume once daily either LNnT or 2'-FL alone (5, 10, 20 g of LNnT or 2'-FL), or a combination of LNnT and 2'-FL (5, 10, 20 g as the total amount of the combination of LNnT and 2'-FL at a ratio of 1:2), or placebo (glucose) for two weeks (unpublished study report, 2015c). At the end of the intervention, a significantly higher incidence of passing gas was reported by participants who consumed either 20 g or 10 g of LNnT compared with the placebo group. No difference to the placebo group was observed in the group with an intake of 5 g LNnT per day. At the end of the intervention, a significantly higher incidence of nausea, rumbling, bloating, passing gas, diarrhoea, loose stools and urgency was reported for participants who consumed 20 g of 2'-FL compared with those in the placebo group. Stool consistency scores on the Bristol Stool scale were not significantly different between the interventions and placebo groups. In total, 44 participants reported adverse events, which could cover more than one symptom, and a total of 78 symptoms were recorded. Gas/flatulence was the most commonly reported adverse event, which was followed by stomach pain, diarrhoea and rumbling. Higher incidence of adverse events was observed in participants who consumed the single oligosaccharide at the highest dose tested (20 g). All adverse events were reported to be 'mild' and no serious adverse events were reported.

3.8. Allergenicity

The manufactured LNnT contains no detectable proteins or peptides (Section 3.1) and so would not be anticipated to elicit any allergenic effect.

The Panel considers that the likelihood of adverse allergic reactions to the NFI is low.

4. Discussion

The NFI is LNnT, a synthetic tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose and D-glucose, which is produced by using D-lactose as a starting raw material. The NFI is intended to be used in IF, FOF, including foods for special medical purposes, and other foods for infants and young children. The NFI is also intended to be used in foods or food supplements for adults.

Considering that LNnT is a naturally occurring tetrasaccharide that is present in human milk, the history of human exposure to LNnT is limited primarily to that of BF infants. The Panel notes that the proposed maximum intake level of the NFI by infants is within the range of intake levels from breast milk reported in the literature.

A large proportion of ingested HMO, including LNnT, reaches the large intestine where it is either fermented by the intestinal microbiota or excreted unchanged in the faeces. There are no indications that the absorption of the NFI may differ from that of LNnT from breast milk and the consumption levels of the NFI would likely be similar to those of LNnT from breast milk by infants. Therefore, the

Panel considers that the limited amount of the NFI that could be absorbed, as suggested by the data provided, does not raise safety concerns.

Three studies were provided on the potential mutagenicity of the NFI: a bacterial reverse mutation assay (Ames test), an *in vitro* mammalian cell gene mutation test and an *in vitro* micronucleus assay. The Panel considers that the information provided does not raise safety concerns as regards the genotoxicity of the NFI.

Based on the observations from a sub-chronic 90-day toxicity study in rats, the Panel considers that the NOAEL is 2 500 mg/kg body weight per day.

The applicant provided an unpublished study report on a double-blind, randomised, controlled, clinical trial on the effects of a combination of two oligosaccharides, LNnT and 2'-FL, which are both under safety assessment as NFIs, in infants. This study on the combination of these two oligosaccharides reported that the weight gain of infants who consumed the formula with added LNnT and 2'-FL was not inferior to the weight gain of infants who were fed the control formula up to four months of age. Mean weight, length, head circumference or BMI in infants aged 0-4 months were comparable to the corresponding WHO standard growth curves. The results on stool endpoints and the reported changes in the composition of microbiota in infants consuming the combination of the two oligosaccharides do not raise safety concerns for the studied combination. The Panel considers that this study can be used to draw conclusions on the safety of this combination at the concentrations tested (i.e. 0.5–0.6 g/L of LNnT and 1.0–1.2 g/L of 2'-FL for reconstituted formula). Based on EFSA food consumption data for infants, such a concentration of LNnT, would result in about 637 mg per day and about 104 mg/kg body weight per day, respectively, at the 95th percentile for a 3-month-old infant weighing 6 kg. According to the intake estimate provided by the applicant based on individual consumption data from the UK, 0.6 g/L LNnT in infant formula would lead to an intake of about 330 mg/kg body weight per day of LNnT at the 95th percentile for infants aged 4-6 months.

Taking into account that human milk contains a large number of oligosaccharides, which all together may have an important role for the development of microbiota in infants, the Panel considers that it cannot be excluded that the addition of a single oligosaccharide, i.e. LNnT alone, to IF (i.e. without 2'-FL) may have different effects on infants' growth, microbiota, and stool frequency and consistency from those observed with the studied combination of the two oligosaccharides added to IF in the provided study. The Panel therefore considers that no conclusion can be drawn from this infant study on the safety of the use of LNnT (without 2'-FL) added to IF.

Noting that complementary feeding of infants and young children results in an increasingly diversified diet, the Panel considers that the potential impact of the addition of LNnT alone (without 2'-FL) or in combination with 2'-FL on the development of the microbiota diminishes with the proportion of complementary feeding and with age. According to the applicant's intake estimate based on UK data, the LNnT intake at the 95th percentile for toddlers corresponds to about 1.9 g per day. On a mg per kg body weight per day basis, the respective value was 132, which is quite below the estimated 95th percentile intake for infants aged 4-6 months based on UK NDNS consumption data. The Panel considers that LNnT alone or in combination with 2'-FL added at the proposed concentration in foods intended for children older than one year of age, does not raise safety concerns. The Panel considers that the concentration of LNnT as applied in the study in infants, should be used in IF, FOF and young-child formula (as described by EFSA (EFSA NDA Panel, 2014)).

The applicant also provided an unpublished study report on a placebo-controlled, double-blind, parallel, dose-response trial in healthy adults who were randomised to consume either LNnT or 2'-FL, alone or in different combinations, or placebo for two weeks. This study showed that participants who consumed daily either 20 g or 10 g of LNnT reported a significant increase in passing gas compared with the placebo group, while, for subjects consuming 5 g per day, this effect was not observed. Considering the highest estimated 95th percentile daily intake of LNnT at 3.3 g and 53 mg/kg body weight, respectively, for women of child-bearing age plus the maximum intended daily intake from food supplements of 1.5 g, daily LNnT intakes in this population group could theoretically result up to 4.8 g per day. The Panel notes that the intake estimate is based on the conservative assumption that all proposed food items consumed by an individual actually contain the NFI at the maximum specified level of use.

5. Conclusions

The Panel concludes that the novel food ingredient, lacto-*N*-neotetraose (LNnT):

- is safe for infants (up to one year of age) when added to infant and follow-on formulae, in combination with 2'-*O*-fucosyllactose (2'-FL), at concentrations up to 0.6 g/L of LNnT and up to 1.2 g/L of 2'-FL, at a ratio of 1:2 in the reconstituted formulae;
- is safe for young children (older than one year of age) when added to follow-on and young child formulae, at concentrations up to 0.6 g/L of LNnT (alone or in combination with 2'-FL, at concentrations up to 1.2 g/L, at a ratio of 1:2);
- is safe when added to other foods at the proposed uses and use levels.

Documentation provided to EFSA

1. Letter from the European Commission to the European Food Safety Authority with the request for a scientific opinion on lacto-*N*-neotetraose (LNnT) as a novel food ingredient. SANCO/E6/SH/ks ref. Ares(2014)3857425, dated 19 November 2014.

2. Dossier 'Application for the approval of the human-identical milk oligosaccharide lacto-*N*-neotetraose (LNnT) as a novel food ingredient for use in infant formulae and in foods' received by EFSA on 1 December 2014. Submitted by Glycom A/S.

3. Initial assessment report carried out by the Food Safety Authority of Ireland: 'Safety assessment of lacto-*N*-neotetraose (LNnT)'.

4. Member States' comments and objections.

5. Response by the applicant to the initial assessment report and the Member States' comments and objections.

6. Additional data were provided by the applicant on 5 and 25 March 2015, 21 and 28 April 2015, 18 May 2015, and 1, 5 and 12 June 2015.

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Abbreviations

2'-FL	2'-*O*-fucosyllactose
BF	breast-fed
CI	confidence interval
DNSIYC	diet and nutrition survey on infants and young children
EMA	European Medicines Agency
FAIM	food additive intakes model
FOF	follow-on formula(e)
FOS	fructo-oligosaccharides
GLP	good laboratory practices
GOS	galacto-oligosaccharides
Hb	haemoglobin
HMO	human milk oligosaccharide
HPLC	high-performance liquid chromatography
IF	infant formula(e)
ITT	intention-to-treat
LNnT	lacto-*N*-neotetraose
NDNS	national diet and nutrition survey
NFI	novel food ingredient
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level
OECD	Organisation for Economic Co-operation and Development
OR	odds ratio
PCV	packed cell volume
PND	post-natal day
PP	per-protocol
UHT	ultra-high temperature
WBC	white blood cell
WHO	World Health Organization