SCIENTIFIC OPINION

EFSA Journal

ADOPTED: 15 May 2019

doi: 10.2903/j.efsa.2019.5717

Safety of 2′-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283

EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA), Dominique Turck, Jacqueline Castenmiller, Stefaan De Henauw, Karen Ildico Hirsch-Ernst, John Kearney, Alexandre Maciuk, Inge Mangelsdorf, Harry J McArdle, Androniki Naska, Carmen Pelaez, Kristina Pentieva, Alfonso Siani, Frank Thies, Sophia Tsabouri, Marco Vinceti, Francesco Cubadda, Karl Heinz Engel, Thomas Frenzel, Marina Heinonen, Rosangela Marchelli, Monika Neuhäuser-Berthold, Annette Pöting, Morten Poulsen, Yolanda Sanz, Josef Rudolf Schlatter, Henk van Loveren, Qingqing Sun, Emanuela Turla and Helle Katrine Knutsen

Abstract

Following a request from the European Commission, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked to deliver an opinion on 2′-fucosyllactose/difucosyllactose (2′-FL/DFL) mixture as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The NF is a powdered mixture mainly composed of two oligosaccharides, 2′-FL and DFL, which are produced together by fermentation with a genetically modified strain of *Escherichia coli* K12. 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 in a variety of foods, including infant and follow-on formula, foods for infants and young children, foods for special medical purposes and food supplements. The target population is the general population except for food supplements, for which the target population is individuals above 1 year of age. Since the intake of 2′-FL and DFL from the NF at the proposed use levels is unlikely to exceed the intake level of naturally occurring 2′-FL and DFL in breastfed infants per kilogram body weight, the Panel concludes that the NF, a mixture of 2′-FL and DFL, is safe under the proposed conditions of use for the proposed target population.

Keywords: 2′-fucosyllactose, difucosyllactose, 2′-FL, DFL, oligosaccharide, novel food, safety

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

Question number: EFSA-Q-2018-00374

Correspondence: nda@efsa.europa.eu

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

Acknowledgements: The Panel wishes to thank Davide Arcella for the support provided to this scientific opinion.

Suggested citation: EFSA NDA Panel (EFSA Panel on Nutrition, Novel Foods and Food Allergens), Turck D, Castenmiller J, De Henauw S, Hirsch-Ernst KI, Kearney J, Maciuk A, Mangelsdorf I, McArdle HJ, Naska A, Pelaez C, Pentieva K, Siani A, Thies F, Tsabouri S, Vinceti M, Cubadda F, Engel KH, Frenzel T, Heinonen M, Marchelli R, Neuhäuser-Berthold M, Pöting A, Poulsen M, Sanz Y, Schlatter JR, van Loveren H, Sun Q, Turla E and Knutsen HK, 2019. Scientific Opinion on the safety of 2'-fucosyllactose/difucosyllactose mixture as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal 2019;17(6):5717, 23 pp. https://doi.org/10.2903/j.efsa.2019.5717

ISSN: 1831-4732

The EFSA Journal is a publication of the European Food Safety Authority, an agency of the European Union.

Summary

Following a request from the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA) was asked to deliver a scientific opinion on 2'-fucosyllactose/difucosyllactose (2'-FL/DFL) mixture as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The assessment of the safety of this NF, which follows the methodology set out in the EFSA Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283 and in the Commission Implementing Regulation (EU) 2017/2469, is based on the data supplied in the application, information submitted by the applicant following the European Food Safety Authority (EFSA) requests for supplementary information and additional data identified by the Panel.

The NF is a mixture mainly composed of two oligosaccharides, 2'-FL and DFL, which are obtained by fermentation with a genetically modified strain of *Escherichia coli* K12. 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 in a variety of foods, including infant and follow-on formula (IF and FOF), foods for infants and young children, foods for special medical purposes and food supplements. The target population is the general population except for food supplements, for which the target population is individuals above 1 year of age.

Considering that 2'-FL and DFL are naturally occurring oligosaccharides present in human milk, the history of human exposure to 2'-FL and DFL concerns breast-fed infants. The Panel notes that 2'-FL, which is the major component of the NF, has already been assessed and authorised as a NF to be added to IF, FOF, to a variety of foods as well as to food supplements.

The Panel considers that there are no concerns regarding genotoxicity of the NF.

The Panels considers that a no observed adverse effect level could not be established from the 90-day oral toxicity study with the NF. However, the intake of 2'-FL and DFL in breastfed infants on a per kg body weight (bw) basis is expected to be safe also for other population groups.

The anticipated daily intake of the NF from the consumption of IF (only), in infants up to 16 weeks of age, does not exceed the high intake level of 2'-FL and DFL in breastfed infants per kg bw. The anticipated daily intake of the NF for the proposed uses at their respective maximum use levels is unlikely to exceed the high intake level of 2'-FL and DFL in breastfed infants per kg bw. The maximum daily intake of the NF as food supplements (i.e. 4 g/day) for individuals above 1 year of age does not exceed the high intake level of 2'-FL and DFL in breastfed infants per kg bw. Thus, the Panel considers that the intake of the NF for the proposed uses at their respective maximum use levels can be considered safe.

The Panel concludes that the NF, a mixture of 2'-FL and DFL, is safe under the proposed conditions of use. The target population is the general population, except for food supplements for which the target population is individuals above 1 year of age. Food supplements are not intended to be used if other foods with added NF or 2'-FL (as well as breast milk for young children) are consumed the same day.

Table of contents

1. Introduction

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

On 30 April 2018, the company Glycom A/S submitted a request to the European Commission in accordance with Article 10 of Regulation (EU) No 2015/2283[1] to place on the EU market 2'-fucosyllactose/difucosyllactose mixture as a novel food (NF). The 2'-fucosyllactose/difucosyllactose mixture is intended to be used in a number of food categories.

In accordance with Article 10(3) of Regulation (EU) 2015/2283, the European Commission asks the European Food Safety Authority to provide a scientific opinion on 2'-fucosyllactose/difucosyllactose mixture as a NF.

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 EFSA requests for supplementary information.

During the assessment, the Panel identified additional data which were not included in the application (Urashima et al., 2013, 2018; Oftedal et al., 2014).

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[2].

A common and structured format on the presentation of NF applications is described in the EFSA guidance on the preparation and presentation of a NF application.[3] As indicated in this guidance, it is the duty of the applicant to provide all available (proprietary, confidential and published) scientific data, including both data in favour and not in favour to supporting the safety of the proposed NF.

This NF application includes a request for protection of proprietary data in accordance with Article 26 of Regulation (EU) 2015/2283. Data claimed to be proprietary by the applicant include:

- annexes to the dossier which relate to the identity, the production process, production microorganism, composition and specifications of the NF (Annex I 'NMR Analytical Reports on Structure Comparison of 2'-FL and DFL from microbial fermentation to breast milk 2'-FL and DFL'; Annex II 'Production Strain Data'; Annex III 'Production Strain Certificates'; Annex IV 'Raw Materials and Processing Aids'; Annex V 'Certificates of Analysis and Batch Data'; Annex VI 'Analytical Methods and Validation Reports'; Annex VII 'Stability Reports'; Annex VIII 'Laboratory Accreditation Certificates';
- 'Intake assessment report' (Annex X to the dossier);
- bacterial reverse mutation test (Unpublished study report, 2017a), *in vitro* micronucleus test (Unpublished study report, 2017b), 14-day and 90-day oral toxicity studies with the NF (unpublished study report, 2017c, 2018, respectively) including the summary table of the statistically significant observations in the 90-day study (Appendix B.3 to the dossier);
- bacterial reverse mutation test (unpublished study report, 2015c), *in vitro* micronucleus tests (unpublished study reports, 2015a,b), and 90-day oral toxicity study with 2'-fucosyllactose (unpublished study report, 2015d).

[1] Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (2013/0435 (COD). OJ L 327, 11.12.2015, p. 1–22.

[2] Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, pp. 64–71.

[3] EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), Turck D, Bresson J-L, Burlingame B, Dean T, Fairweather-Tait S, Heinonen M, Hirsch-Ernst KI, Mangelsdorf I, McArdle H, Naska A, Neuhäuser-Berthold M, Nowicka G, Pentieva K, Sanz Y, Siani A, Sjödin A, Stern M, Tomé D, Vinceti M, Willatts P, Engel K-H, Marchelli R, Pöting A, Poulsen M, Salminen S, Schlatter J, Arcella D, Gelbmann W, de Sesmaisons-Lecarré A, Verhagen H and van Loveren H, 2016. Guidance on the preparation and presentation of an application for authorisation of a novel food in the context of Regulation (EU) 2015/2283. EFSA Journal 2016;14(11):4594, 24 pp. https://doi.org/10.2903/j.efsa.2016.4594

2.2. Methodologies

The assessment follows the methodology set out in the EFSA guidance on NF applications 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.

This assessment concerns only 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 2'-fucosyllactose/difucosyllactose mixture with regard to any claimed benefit.

3. Assessment

3.1. Introduction

The NF is a mixture of 2'-fucosyllactose (2'-FL) and difucosyllactose (DFL) obtained by microbial fermentation using D-lactose and D-glucose as raw materials. The NF is intended to be used in foods for infants and young children, foods for special medical purposes, total diet replacements for weight control, food supplements and various foods. The target population is the general population (except for food supplements which is individuals above 1 year of age). The applicant indicated that this 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 two oligosaccharides, 2'-FL and DFL, which are produced together by fermentation with a genetically modified strain of *Escherichia coli* K12. 2'-FL is a trisaccharide consisting of D-lactose and L-fucose, linked at the D-galactose moiety by an α(1→2) bond (chemical formula: $C_{18}H_{32}O_{15}$; molecular mass: 488.44 Da; CAS No 41263-94-9). DFL is a tetrasaccharide, where a second L-fucose moiety has been added to the 3-position of D-glucose in 2'-FL by an α(1→3) bond (chemical formula: $C_{24}H_{42}O_{19}$; molecular mass: 634.58 Da; CAS No 20768-11-0).

The relative structures have been confirmed by monodimensional (1D) and two-dimensional (2D NOESY) ^{1}H-nuclear magnetic resonance spectroscopy (NMR) and by mass spectrometry (MS). The structures are consistent with what is reported in the literature (Oftedal et al., 2014; Urashima et al., 2018). The 2'-FL and DFL obtained by microbial fermentation are demonstrated to be chemically and structurally identical to the oligosaccharides present in human milk (Pratico et al., 2014).

3.3. Production process

The NF is produced according to Good Manufacturing Practice (GMP) and Hazard Analysis Critical Control Points (HACCP) principles.

The manufacturing process can be broadly divided into two stages. In the first stage, D-lactose and D-glucose are converted to 2'-FL and DFL by the modified cellular metabolism of the production microorganism, which uses D-glucose as an exclusive energy and carbon source and D-lactose as a substrate for the biosynthesis of 2'-FL and DFL. The production microorganism, which is a genetically modified derivative of *E. coli* K-12, is entirely removed from the medium at the end of the fermentation process (ultrafiltration/diafiltration). The second stage of the process consists of a series of purification, concentration and isolation steps to obtain the NF as a spray-dried powdered mixture.

The production strain used in the fermentation is a genetically modified derivative of *E. coli* K-12 DH1. The parental strain *E. coli* K-12 DH1 (λ-gyrA96 recA1 relA1 endA1 thi-1 hsdR17 supE44) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and deposited under DSM No. 4235. Although the species *E. coli* was considered not suitable for qualified presumption of safety (QPS) status (EFSA BIOHAZ Panel, 2018), *E. coli* K-12 is considered as an example of safe and non-pathogenic or toxigenic microorganism widely used for biotechnological applications (Gorbach, 1978; OECD, 1986; Muhldorfer and Hacker, 1994; U.S. EPA, 1997). The whole genomes of *E. coli* K-12 and of other derivative strains, including *E. coli* K12 DH1, were sequenced and compared with other *E. coli* strains. The results indicate that *E. coli* K-12 and its derivatives show genomic differences compared to pathogenic strains (Blattner et al., 1997; Lukjancenko et al., 2010).

The genetically modified derivative of the *E. coli* K-12 DH1 strain, which is used in the production process of the NF, has been deposited in the DSMZ in Braunschweig, Germany, under DSM No. 32774. The applicant provided a detailed description of the genetic modification process applied to the parental strain to obtain the platform strain *E. coli* K-12 DH1 MDO (membrane-derived oligosaccharide) and to the final production microorganism.

The absence of the production microorganism in the NF was demonstrated by testing five batches of the NF for bacteria from the Enterobacteriaceae family according to internationally recognised methods (ISO 21528-1:2004, MSZ ISO 21528-2:2007). Upon EFSA's request for additional information, the applicant provided data to demonstrate the absence of DNA from the genetically modified production microorganism in the NF in accordance to the EFSA guidance on microorganisms used 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

The applicant provided batch-to-batch composition for five batches of the NF (Table 1), analysed using high-performance anion exchange chromatography coupled to pulsed amperometric detection (HPAEC-PAD).

The NF is a mixture mainly composed of two oligosaccharides, 2'-FL and DFL (81% and 11%, respectively, as the average from five batches of the NF), accompanied by other carbohydrates (e.g. D-lactose, L-fucose, 2'-L-fucosyl-D-lactulose). Together with other related saccharides naturally occurring in milk (i.e. lactose, fucose), the 2'-FL/DFL mixture comprises 94% of the total weight (mean from five batches of the NF). The remaining portion of the product consists mainly of other carbohydrate-type compounds structurally related to 2'-FL and DFL. Non-carbohydrate residues including amino acids, amino acid metabolites, biogenic amines, microbial endotoxins, residual proteins, anions, trace elements and heavy metals were analysed and not detected in the purified product.

The microbiological purity of batches of the NF has been assessed with regard to non-pathogenic microorganisms (bacteria, yeasts and moulds) as general hygiene indicators, and selected food-borne pathogens.

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

Table 1: Batch-to-batch analysis for five batches of NF

Parameters	Batches					
	CPN6317 1000417 FD	**CPN6317 1000517 FD**	**CPN6317 1000717 FD**	**CPN6317 1000917 FD**	**CPN6317 1001017 FD**	**Mean ± Standard Deviation**
Physicochemical properties						
Appearance	Powder or agglomerates					
Colour	White to off white					
pH (20°C, 5% solution)	4.5	4.4	4.6	4.1	4.8	4.5 ± 0.3
Composition						
Sum of 2'-FL, DFL, lactose, and fucose [w/w % dry matter]	93.6	93.2	93.9	94.0	94.4	94 ± 0.4
Sum of 2'-FL and DFL [w/w % dry matter]	90.6	92.1	92.6	90.3	93.7	92 ± 1
2'-FL [w/w % dry matter]	79.1	82.5	81.7	78.8	81.7	81 ± 2
DFL [w/w % dry matter]	11.5	9.6	10.9	11.5	12.0	11 ± 1
Ratio 2'-FL:DFL	6.9	8.6	7.5	6.9	6.8	7 ± 1
D-Lactose [w/w %]	2.98	0.96	1.29	3.56	0.64	1.9 ± 1.3
L-Fucose [w/w %]	0.05	0.06	0.04	0.09	0.06	0.1 ± 0.0
2'-Fucosyl-D-lactulose [w/w %]	1.1	0.9	0.8	0.8	0.9	0.9 ± 0.1
Sum of other carbohydrates(1) [w/w %]	2.9	2.4	2.1	2.0	2.2	2.3 ± 0.1

Parameters	Batches					
	CPN6317 1000417 FD	**CPN6317 1000517 FD**	**CPN6317 1000717 FD**	**CPN6317 1000917 FD**	**CPN6317 1001017 FD**	**Mean ± Standard Deviation**
Water [w/w %]	0.38	0.44	0.42	0.44	0.47	0.43 ± 0.03
Ash, sulfated [w/w %]	0.02	0.06	0.03	0.01	0.07	0.04 ± 0.03
Microbiological parameters						
Aerobic mesophilic total plate count [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10
Enterobacteriaceae [in 10 g]	Absent	Absent	Absent	Absent	Absent	Absent
Salmonella spp. [in 25 g]	Absent	Absent	Absent	Absent	Absent	Absent
Cronobacter (Enterobacter) sakazakii [in 10 g]	Absent	Absent	Absent	Absent	Absent	Absent
Listeria monocytogenes [in 25 g]	Absent	Absent	Absent	Absent	Absent	Absent
Bacillus cereus [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10
Yeasts [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10
Moulds [CFU/g]	< 10	< 10	< 10	< 10	< 10	< 10

2'-FL: 2'-fucosyllactose; CFU: colony forming units; DFL: difucosyllactose.
(1): Sum of carbohydrates such as 3'-fucosyllactose (3'-FL), 2'-fucosyl-galactose, glucose, galactose, mannitol, sorbitol, galactitol, trihexose, allo-lactose and other structurally related carbohydrates.

3.4.1. Stability

Stability of NF

The stability of the NF has been investigated in two representative batches in an ongoing 5-year study under real-time conditions (25°C, 60% relative humidity (RH)) and in an ongoing 2-year study under accelerated conditions (40°C, 75% RH). Upon EFSA's request for additional information, the applicant provided 12-month interim results of two batches of the NF from these studies. Results on composition, physical and microbiological parameters have been provided. The results indicate that the NF is stable when stored at room temperature for at least 12 months.

Stressed/forced stability tests with the NF as amorphous powder and in aqueous solutions, at acidic or neutral pH, and stored at 60 and 80°C for 8 and 4 weeks, respectively, have been performed. The results of these studies indicated the presence of two potential pH-dependent chemical pathways in the aqueous solutions of the NF, i.e. hydrolysis at pH < 5.0 and isomerisation at pH > 6.0. At neutral pH, 2'-FL and DFL underwent minor isomerisation to fucosyl-lactulose and difucosyl-lactulose, respectively.

The applicant also referred to stability studies on 2'-FL alone. A 2-year accelerated stability study (40°C, 75% RH) on the chemically synthesized 2'-FL (crystalline form) demonstrated that 2'-FL is stable for 3 years, when stored at room temperature (EFSA NDA Panel, 2015).

The applicant provided a 5-year real time stability study on one batch of 2'-FL (amorphous powder), at room temperature, which confirms the 5 years shelf-life of the amorphous 2'-FL upon storage at 25°C.

The Panel considered that the available data provide sufficient information with respect to the stability of the NF.

Stability of NF under the intended conditions of use

The stability of the NF in powdered infant formula has been investigated when stored at 4, 20, 30 and 37°C up to 6 months.

The infant formula powder tested was a whey-based commercially available formula supplemented with the NF. No appreciable losses were observed for 2'-FL and DFL at the time points tested (3 and 6 months).

3.5. Specifications

The specifications of the NF as proposed by the applicant are presented in Table 2. The parameters include the main components of the mixture (2'-FL and DFL), the raw material D-lactose and possible degradation products (L-fucose and 2'-L-fucosyl-D-lactulose).

Microbiological parameters for *Listeria monocytogenes*, *Cronobacter (Enterobacter) sakazakii* and *Bacillus cereus* are monitored through internal specifications.

Analyses were performed using internationally recognised methods or newly developed and validated analytical protocols at Glycom's Research & Development Department and confirmed by accredited laboratories.

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

Table 2: Specifications of the NF

Description: The 2′-fucosyllactose/difucosyllactose (2'-FL/DFL) mixture is a purified, white to off-white amorphous powder that is produced by a microbial process. After purification, the 2′-FL/DFL powdered mixture is obtained by spray drying		
Source: A genetically modified strain of *Escherichia coli* K-12 DH1 DMO		
Parameter	**Specification**	**Method**
Appearance	Powder or agglomerates	ISO 6658:2007
Colour	White to off white	ISO 6658:2007
Identification (2'-FL/DFL mixture)	RT of standards ± 3%	HPAEC/PAD
Sum of 2'-FL, DFL, lactose and fucose [% dry matter]	≥ 92.0 w/w %	HPAEC/PAD
Sum of 2'-FL and DFL [% dry matter]	≥ 85.0 w/w %	HPAEC/PAD
2'-FL [% dry matter]	≥ 75.0 w/w %	HPAEC/PAD
DFL [% dry matter]	≥ 5.0 w/w %	HPAEC/PAD
D-Lactose	≤ 10.0 w/w %	HPAEC/PAD
L-Fucose	≤ 1.0 w/w %	HPAEC/PAD
2'-Fucosyl-D-lactulose	≤ 2.0 w/w %	HPAEC/PAD
Sum of other carbohydrates(1)	≤ 6.0 w/w %	HPAEC/PAD
pH (20°C, 5% solution)	4.0–6.0	Eur. Ph. 9.2 2.2.3 (07/2016:20203)
Water	≤ 6.0 w/w %	Karl-Fischer
Ash, sulfated	≤ 0.8 w/w %	Eur. Ph. 9.2 2.4.14 (04/2010:20414)
Residual protein	≤ 0.01 w/w %	Bradford assay (UV spectroscopy)(2)
Microbiological Parameters		
Aerobic mesophilic total plate count	≤ 1000 CFU/g	ISO 4833-1:2014
Enterobacteriaceae	≤ 10 CFU/g	ISO 21528-1:2004, ISO 21528-2:2007
Salmonella spp.	Absent in 25 g	ISO 6579:2006
Yeasts	≤ 100 CFU/g	ISO 7954:1999
Moulds	≤ 100 CFU/g	ISO 7954:1999
Residual endotoxins	≤ 10 EU/mg	Eur. Ph. 2.6.14

2'-FL: 2'-fucosyllactose; CFU: colony forming units; DFL: difucosyllactose; EU: endotoxin units; Eur. Ph.: European Pharmacopoeia; HPAEC/PAD: high-performance anion exchange chromatography/pulsed amperometric detection; RT: retention time; UV: ultraviolet.

(1): Sum of carbohydrates such as 3'-fucosyllactose (3'-FL), 2'-fucosyl-galactose, glucose, galactose, mannitol, sorbitol, galactitol, trihexose, allo-lactose and other structurally related carbohydrates.

(2): LOR = 17 mg/kg.

3.6. History of use of the NF

3.6.1. History of use of the NF

The NF as such does not have a history of use.

However, 2'-FL, which is the major constituent of this NF, has been authorised as a NF in the European Union (Commission Implementing Regulation 2018/1023[4]) to be added to infant and follow-on formulae (IF, FOF), to a variety of foods as well as to food supplements. 2'-FL is obtained either from fermentation with genetically modified *E. coli* strains (K12 or BL21) or by chemical synthesis. The production of these already authorised 2'-FL results in the presence of DFL (up to 5%) in the final product.

2'-FL has also been authorised to be used as a food ingredient in USA and Singapore (FDA, 2015, 2016; AVA, 2018).

3.6.2. Consumption of 2'-FL and DFL in breast milk

Human breast milk contains a family of structurally related oligosaccharides, known as human milk oligosaccharides (HMOs), which constitute the third main component in human milk after lactose and fats. 2'-FL and DFL belong to the group of fucosylated HMOs, which constitute on average around 70% of the total HMO fraction in human milk (Bode, 2012).

Several publications on 2'-FL and DFL in human milk have been provided by the applicant. The amount of 2'-FL and DFL in human milk depends on the lactation period, with higher levels reported in colostrum (Coppa et al., 1999, 2011; Erney et al., 2000; Morrow et al., 2004; Musumeci et al., 2006; Asakuma et al., 2008, 2011; Thurl et al., 2010, 2017; Galeotti et al., 2012, 2014; Spevacek et al., 2015; Austin et al., 2016; McGuire et al., 2017). Erney et al. (2001) reported mean concentrations of 2.38 g/L for 2'-FL and 0.46 g/L for DFL, in pooled human milk samples from different lactating periods, with high occurrence levels of 4.78 g/L for 2'-FL and 2.44 g/L for DFL reported in individual samples.

Based on the mean and high occurrence levels of 2'-FL and DFL in human milk as reported by Erney et al. (2001), and considering the average and high daily intake of breast milk (800 mL and 1,200 mL, respectively) for infants from 0 to 6 months (EFSA NDA Panel, 2013), the NDA Panel calculated the daily intake levels of 2'-FL and DFL per kg body weight (bw) from human milk for an infant with a default bw of 6.7[5] kg (Table 3). The default bw used by the NDA Panel is for an infant of 3–6 months of age, who is more likely to consume such amounts of human milk.

Oligosaccharides, including 2'-FL and DFL, have also been detected in domestic farm animal milk, however, at lower concentrations as compared to human milk (Aldredge et al., 2013; Urashima et al., 2013; Albrecht et al., 2014).

Table 3: Estimated daily intake levels of 2'FL and DFL from human milk (800 mL and 1,200 mL) for infants of 6.7 kg bw, based on mean and high concentration of 2.38 g/L and 4.78 g/L, respectively, of 2'-FL and mean and high concentration of 0.46 g/L and 2.44 g/L, respectively, of DFL in human milk

	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**
2'FL	284	571	426	856
DFL	55	291	82	437

2'-FL: 2'-fucosyllactose; DFL: difucosyllactose bw: body weight.

[4] Commission Implementing Regulation (EU) 2018/1023 of 23 July 2018 correcting Implementing Regulation (EU) 2017/2470 establishing the Union list of novel foods. Official Journal of the European Union L 187, 1–133.

[5] EFSA Scientific Committee, 2012.

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, except for food supplements for which the target population is individuals above 1 year of age.

3.7.2. Proposed uses and use levels

The NF is intended to be added to a variety of foods, at the maximum use levels as indicated in Table 4. The Panel notes that for 'Foods for special medical purposes' the applicant did not propose either maximum use levels or maximum intake levels.

The applicant also intends to market the NF as food supplements, at the maximum daily intake of 4 g, for individuals above 1 year of age.

Table 4: Proposed food uses and maximum use levels of the NF

EU food category number	Food category name	Proposed maximum use level of the NF
1	**Dairy products and analogues**	
1.1	Unflavoured pasteurised and unflavoured sterilised (including UHT) milk	2.0 g/L
1.2/1.3	Unflavoured fermented milk-based products	2.0 g/L beverages
		20 g/kg products other than beverages
1.4	Flavoured fermented milk-based products including heat-treated products	2.0 g/L beverages
		20 g/kg products other than beverages
7	**Bakery wares**	
7.2	Fine bakery wares. **Cereal bars only**	20 g/kg
13	**Foods for Special Groups**	
13.1	**Foods for infants and young children**	
13.1.1	Infant formula as defined in Regulation (EU) No 609/2013	1.6 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
13.1.2	Follow-on formula as defined in Regulation (EU) No 609/2013	1.2 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
13.1.3	Processed cereal-based food and baby food for infants and young children as defined in Regulation (EU) No 609/2013	1.2 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
		10 g/kg for products other than beverages
13.1.4[13]	Milk-based drinks and similar products intended for young children	1.2 g/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
		10 g/kg for products other than beverages
13.2	**Foods for special medical purposes as defined in Regulation (EU) No 609/2013**	
13.2	Foods for special medical purposes as defined in Regulation (EU) No 609/2013	On case-by-case basis
13.3	**Total diet replacement for weight control as defined in Regulation (EU) No 609/2013**	
13.3	Total diet replacement for weight control as defined in Regulation (EU) No 609/2013	4.0 g/L beverages
		40 g/kg products other than beverages
14	**Beverages**	
14.1.4	Flavoured drinks	2.0 g/L

UHT: ultra-high temperature.

3.7.3. Anticipated intake of the NF

Anticipated intake of the NF from the consumption of IF in infants up to 16 weeks of age

IF is expected to be the only food consumed by infants aged 0–16 weeks. The high consumption of IF has been estimated to be 260 mL/kg bw per day for infants aged 0–16 weeks (EFSA Scientific Committee, 2017). Based on the maximum proposed use level of the NF (1.6 g/L in IF), the high intake of the NF from IF alone is estimated to be 416 mg/kg bw per day.

Considering the mean content of 81% and 11% for 2'-FL and DFL, respectively, in the NF (as indicated in Table 1), the daily intake of 2'-FL and DFL from the consumption of IF added with the NF corresponds to 337 mg/kg bw for 2'-FL and 46 mg/kg bw for DFL, respectively. The Panel notes that the anticipated daily intake of the NF from the consumption of IF (only) does not exceed the estimated high daily intake of 2'-FL and DFL in breast-fed infants per kg bw (Table 3). The Panel notes that the proposed maximum use level of 2'-FL from the NF in IF (i.e. 1.6 g/L) is in line with the maximum use level already authorised for 2'-FL in IF.[4]

Anticipated intake of the NF from the proposed food uses

The applicant estimated the daily intake of the NF by using the EFSA Food Additive Intake model (FAIM) tool (FAIM 2.0, 2017). However, since the food categories in the FAIM tool, which are based on Regulation (EC) 1333/2008, do not allow a precise matching with the food categories proposed for the NF, the intake estimations performed by the applicant resulted in high and uncertain estimated intakes. Thus, EFSA performed a refined estimate of the anticipated daily intake of the NF, at the maximum proposed use levels of the NF, using individual data from the EFSA Comprehensive Food Consumption Database (EFSA, 2011) and by applying the FoodEx2 classification system (Table 5). The lowest and highest mean and 95th percentile anticipated daily intake of the NF for all subjects, among the EU dietary surveys, are presented in Table 6. The refined anticipated daily intake of the NF for each population group from each EU dietary survey is available in the excel file annexed to this scientific opinion (under supporting information).

Anticipated intake of 2'-FL from the proposed uses and use levels of the NF

The Panel notes that all proposed uses of the NF have already been authorised for a NF which consists of 2'-FL.[4] Considering 81% 2'-FL content in the NF, the maximum proposed use levels of 2'-FL from the NF under assessment are in line with the maximum use levels authorised for 2'-FL.[4]

The Panel also notes that the highest estimated 95th percentile intake of 2'-FL from the NF (i.e. 1,110 mg kg bw = 81% of 1,370 mg NF per kg bw) on the basis of 11 dietary surveys covered by the EFSA Comprehensive Food Consumption Database is about 30 % above the high estimate for 2'-FL from human milk (i.e. 856 mg/kg bw). Considering that the exposure of 2'-FL from human milk is only exceeded in one out of the 11 dietary surveys included in the EFSA Comprehensive Food Consumption Database and the conservative assumption underlying this type of exposure assessment, in particular, assuming that all foods of the proposed food categories consumed by infants are added with the NF at the maximum proposed use levels, the Panel considers that it unlikely that infants would exceed high intake levels of 2'-FL from human milk per kg bw.

Anticipated intake of DFL from the proposed uses and use levels of the NF

Based on 11% DFL content in the NF, the anticipated daily intake of DFL is calculated from the highest 95th percentile anticipated daily intake of the NF (Table 7). The Panel notes that the highest anticipated 95th percentile daily intake of DFL from the NF when added to all food categories listed in Table 5, at the maximum proposed use levels, does not exceed for any group of the general population the high daily intake of DFL in breastfed infants per kg bw (Table 3).

Table 5: FoodEx2 categories and maximum use levels of the NF used in the refined intake of the NF using individual data from EU dietary surveys

CODE	FoodEx2 Level	Food category	Maximum use level of the NF mg/100 g
A02LV	5	Cow milk	200
A0CXA	5	European buffalo milk	200
A02MC	5	Sheep milk	200

CODE	FoodEx2 Level	Food category	Maximum use level of the NF mg/100 g
A02MB	4	Goat milk	200
A02MV	3	Butter milk	200
A02NQ	4	Yoghurt drinks	200
A02NR	4	Probiotic milk-like drinks	200
A02NV	5	Kefir	200
A02NE	4	Yoghurt	2,000
A00EY	4	Cereal bars	2,000
A00EZ	4	Cereal bars plain	2,000
A00FA	4	Cereal bars mixed	2,000
A03PZ	4	Infant formulae, powder	1,300
A03QE	4	Infant formulae, liquid	160
A03QK	4	Follow-on formulae, powder	970
A0EQL	4	Follow-on formulae, liquid	120
A03QZ	3	Cereals with an added high protein food which have to be reconstituted	600
A03QY	3	Simple cereals which have to be reconstituted	600
A0BZF	3	Cereals with added high protein food reconstituted	120
A0BZE	3	Simple cereals for infants and children reconstituted	120
A03RA	3	Biscuits, rusks and cookies for children	1,000
A03RC	2	Ready-to-eat meal for infants and young children	1,000
A03RB	3	Pasta for children	1,000
A03RN	3	Fruit and vegetable juices and nectars specific for infants and young children	120
A03RP	3	Special food for children's growth	120
A03RT	4	Total daily diet replacement for weight reduction	4,000
A0EQN	5	Soft drinks with minor amounts of fruits or flavours	200
A03A	5	Soft drink with fruit juice (fruit content below the minimum for nectars)	200
A03EX	5	Soft-drink, flavoured, no fruit	200
A03FQ	4	Cola type drinks	200

NF: novel food.

Table 6: Ranges among EU surveys of the estimated daily intake of the NF (mg/kg bw), based on the individual data from the EFSA Comprehensive Food Consumption Database

Age groups	Number of EU dietary surveys	Estimated daily intake of the NF – all subjects (mg/kg bw)	
		Range of means (lowest and highest) among EU dietary surveys	Range of 95th percentile (lowest and highest) among EU dietary surveys
Infants (4–11 months)	11	65–418	164–1,370
Young children or toddlers (12–35 months)	14	71–309	230–860
Other children (3–9 years)	19	35–185	95–467
Adolescents (10–17 years)	18	14–48	37–122
Adults (18–64 years)	19	8–29	34–90

Age groups	Number of EU dietary surveys	Estimated daily intake of the NF – all subjects (mg/kg bw)	
		Range of means (lowest and highest) among EU dietary surveys	Range of 95th percentile (lowest and highest) among EU dietary surveys
Elderly (≥ 65 years)	18	8–26	31–74
Pregnant women	2	5–24	14–75
Lactating women	2	20-28	74-77

NF: novel food; bw: body weight.

Table 7: Estimated daily intake of DFL (mg/kg bw) from the highest 95th percentile of intake of the NF, based on 11% of DFL in the NF

Age groups	Estimated daily intake (mg/kg bw)	
	For the NF Highest 95th percentile among EU dietary surveys (as reported in table 6)	For DFL Highest 95th percentile among EU dietary surveys
Infants (4–11 months)	1,370	151
Young children or toddlers (12–35 months)	860	95
Other children (3–9 years)	467	51
Adolescents (10–17 years)	122	13
Adults (18–64 years)	90	10
Elderly (≥ 65 years)	74	8
Pregnant women	75	8
Lactating women	77	8

DFL: difucosyllactose; NF: novel food; bw: body weight

Anticipated intake of the NF from food supplements

The applicant has proposed a maximum daily intake of 4 g of the NF as food supplements for individuals above 1 year of age. Food supplements are not intended to be used if other foods with added NF or 2'-FL are consumed the same day. For young children (aged 12–35 months), food supplements are not intended to be used if breast milk or other foods with added NF or 2'-FL are consumed the same day.

Considering the mean content of 81% and 11% for 2'-FL and DFL, respectively, the maximum daily intake of the NF (i.e. 4 g/day) results in a maximum daily intake of 272 mg/kg bw and 37 mg/kg bw of 2'-FL and DFL, respectively, for young children with bw of 11.9 kg (default body weight value indicated by the EFSA Scientific Committee for young children of 12–35 months of age (2012)). For individuals above 3 years of age, the maximum daily intake of the NF from food supplements in mg per kg bw would be lower than that for young children. The Panel notes that the maximum proposed daily intake of the NF as food supplements (i.e. 4 g/day) for individuals above 1 year of age does not exceed the high daily intake of 2'-FL and DFL from human milk per kg bw for infants (Table 3).

3.7.4. Combined intake from the NF and other sources

As indicated in Section 3.6.1, 2'-FL, which is the major component of the NF under assessment, has been authorised as a NF. The Panel notes that more uses than those proposed for the NF under assessment have been authorised for 2'-FL (i.e. dairy analogues; table-top sweeteners; bread and pasta products gluten-free; coffee/tea/herbal/fruit infusions).

Considering that DFL can be potentially present up to a maximum limit of 5% in the already authorised 2'-FL, the additional authorised uses for 2'-FL which were listed above constitute an additional source of intake of DFL. Overall, the contribution to the DFL intake from the additional already authorised uses of 2'-FL can be considered low (i.e. less than 5% of the highest 95th percentile intake).

Food supplements are not intended to be used if other foods with added NF or 2'-FL (as well as breast milk for young children) are consumed the same day.

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

HMOs, including fucosyllactoses, are considered 'non-digestible oligosaccharides' (EFSA NDA Panel, 2014). HMOs, such as 2'-FL and DFL, do not undergo any significant digestion in the upper gastrointestinal tract (Brand-Miller et al., 1995, 1998; Engfer et al., 2000; Gnoth et al., 2000; Chaturvedi et al., 2001b; 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. (2001b) and Coppa et al. (2001) reported that 97% and 40–50% of the ingested HMOs are excreted unchanged in faeces in breast-fed infants. Approximately 1–2% of the ingested amounts of HMOs are excreted unchanged in the infants' urine.

There are no reasons to assume that the absorption of 2'-FL and DFL, which are the main constituents of the NF, may differ from the absorption of 2'-FL and DFL from human milk.

The Panel considers that limited digestion of the NF occurs in the upper gastrointestinal tract and that only small amounts are expected to be absorbed.

3.9. Nutritional information

The NF is mainly composed of two non-digestible oligosaccharides, i.e. 2'-FL and DFL. The Panel considers that consumption of the NF at the proposed use levels is not nutritionally disadvantageous.

3.10. Toxicological information

The list of toxicological studies, which were provided and claimed proprietary by the applicant, is provided in Table 8. These studies were conducted with the NF (batch no CPN6317 1000517 FD), which contained 82.5% of 2'-FL and 9.64% of DFL.

The applicant also provided toxicological studies on 2'-FL (alone), which was chemically synthesized or produced using different genetically modified strains of *E. coli* (BL21 or K-12) than the one used to produce the NF. The Panel considers that these toxicological studies on 2'-FL can provide supporting evidence for the safety assessment of the NF.

Table 8: List of toxicological studies with the NF provided by the applicant

Test material	Reference	Type of study
NF (82.5% 2'-FL and 9.64% DFL)	Unpublished study report (2017a); Phipps et al. (2018)	Bacterial reverse mutation test (Ames test)
	Unpublished study report (2017b); Phipps et al. (2018)	*In vitro* mammalian cell micronucleus test
	Unpublished study report (2017c)	14-day repeated dose oral toxicity study
	Unpublished study report (2018); Phipps et al. (2018)	90-day repeated dose oral toxicity study

3.10.1. Genotoxicity

The potential genotoxicity of the NF was investigated in a bacterial reverse mutation test and an *in vitro* mammalian cell micronucleus test (Unpublished study report, 2017a,b, Phipps et al., 2018). These studies were conducted in compliance with Organisation for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) (OECD, 1998a) and in accordance with the test guidelines No 471 and 487 from OECD (1997, 2014).

In the bacterial reverse mutation test, *Salmonella* Typhimurium TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2 uvrA (pKM101) were exposed to the NF at concentrations up to 5,000 μg/plate

(either in the absence or presence of S9 mix). No cytotoxicity was observed up to the highest concentration tested. No biologically relevant increase in the number of revertant colonies was observed (with or without S9-mix) up to the highest concentrations tested. The NF is non-mutagenic in this assay.

In the *in vitro* mammalian cell micronucleus test, human lymphocytes were exposed to the NF at concentrations up to 2,000 μg/mL (either in the absence or presence of S9 mix) in both a preliminary and main test. No cytotoxicity was observed up to the highest concentration tested. There was no statistically significant increase in the number of micronucleated cells (with or without S9-mix). The NF is neither clastogenic nor aneugenic in this assay.

Based on the results of these studies, the Panel considers that there are no concerns regarding genotoxicity of the NF.

3.10.2. Repeated dose toxicity studies

The applicant provided a 14-day repeated dose oral toxicity study in Crl:CD(SD) neonatal rats of 7 days of age (8/sex per group) which were administered by gavage 0, 4,000 or 5,000 mg/kg bw per day of the NF (Unpublished study report, 2017c). No claim for compliance with GLP was made. One male in the 5,000 mg/kg bw group was found dead at the end of the treatment period. The cause of the death could not be determined. Transient clinical signs were observed in some animals in both treated groups (i.e. red and/or yellow staining around the anus) and were considered non-adverse. No test-item related effects were observed in this study (i.e. body weight and macroscopic abnormalities). Thus, the authors concluded that the highest dose tested (i.e. 5,000 mg/kg bw per day) would be acceptable as the high-dose level for a 90-day toxicity study in neonatal rats.

The applicant provided a repeated dose toxicity study in which Crl:CD(SD) neonatal rats of 7 days of age (10/sex per group) were administered by gavage either 0, 1,000, 3,000 or 5,000 mg/kg bw per day of the NF, or 5,000 mg/kg bw per day of oligofructose for 90 days (Phipps et al., 2018; Unpublished study report, 2018). Additional groups (5/sex per group) administered by gavage 0 or 5,000 mg/kg bw per day of the NF, or 5,000 mg/kg bw per day of oligofructose for 90 days were followed for a 4-week treatment-free recovery period. This study was conducted in compliance with OECD principles of GLP (OECD, 1998a) and in accordance to the principles and methods described in the test guideline No 408 from OECD (1998b) and in the EMA guideline on repeated dose toxicity (EMA, 2010), with the exception of the age of rats.

Statistically significant differences in some parameters, between the treated and the control groups, have been observed (see Appendix A to this opinion).

Mean activity count in high-dosed females was statistically significantly lower than controls. As this effect was slight and only observed in females, and owing to a no dose–response relationship, this effect it is considered unrelated to the test material.

Mean age and body weight at balano-preputial skinfold separation were slightly higher in high-dosed males as compared with controls (only age was statistically significantly higher and not body weight). However, these differences, which were minor and probably related to low values in the control group compared to historical controls, are not considered to be adverse.

The statistically significant differences observed in several haematological and clinical chemistry parameters in the treated groups as compared to control groups showed an unusual dose–response relationship, where significant changes often were seen only in the low- and mid-dose group. Differences observed in the high-dose group were minor or not statistically significant compared to the control group. No macroscopic or histopathological findings were seen in the treated groups.

Statistically significant changes in urinalysis parameters and in some relative organ weights, which were of low magnitude and occurred only in one gender, are not considered by the Panel to be treatment-related.

The NDA Panel notes that the treatment of animals by gavage already prior to weaning in the 90-day study in rats, applying a dose range of up to 5,000 mg/kg bw per day of the NF, may induce variability in some outcomes. The Panel could not interpret the biological relevance and adversity of the significant differences in the observed haematological and clinical chemistry parameters, which did not show a dose–response relationship. For this reason and although other parameters did not show toxicologically relevant differences, the Panel could not establish a no observed adverse effect level (NOAEL) from this 90-day oral toxicity study with the NF.

3.10.3. Human data

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

The applicant referred to four human studies which have been performed with 2'-FL (alone) obtained via chemical synthesis. In these randomised, double-blind, controlled intervention studies 2'-FL was administered either alone or in combination with lacto-*N*-neotetraose or other oligosaccharides either to infants or adults (Marriage et al., 2015; Elison et al., 2016; Kajzer et al., 2016; Puccio et al., 2017). In particular, the study by Elison et al. (2016), which was previously assessed by the Panel (EFSA NDA Panel, 2015), reported a statistically significant increase in gastrointestinal symptoms (nausea, rumbling, bloating, passing gas, diarrhoea, loose stools and urgency) in adults consuming 20 g/day of 2'-FL for 2 weeks as compared with the placebo group.

3.11. Allergenicity

The protein content in the NF is below 0.01% (w/w) (limit of reporting (LOR) = 17 mg/kg) as indicated in the specifications (Table 2). The applicant provided evidence for the absence of the production microorganisms and residual DNA in the NF.

In addition, the applicant has assessed the allergenic potential of introduced proteins as a result of the genetic modification of the *E. coli* K-12 host (which itself is recognised as non-allergenic) using the search algorithms provided by the Allergen Online tool (version 17) of the University of Nebraska (FARRP, 2017). No sequence alerts for potential allergenicity were identified.

The Panel considers that the likelihood of adverse allergenic reactions to the NF is very low.

4. Discussion

The NF is a mixture mainly composed of two oligosaccharides, 2'-FL and DFL, which are obtained by fermentation with a genetically modified strain of *E. coli* K12. 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 in a variety of foods, including IF and FOF, foods for infants and young children, foods for special medical purposes and food supplements. The target population is the general population except for food supplements, for which the target population is individuals above 1 year of age.

Considering that 2'-FL and DFL are naturally occurring oligosaccharides present in human milk, the history of human exposure to 2'-FL and DFL concerns breast-fed infants. The Panel notes that 2'-FL, which is the major component of the NF, has already been assessed and authorised as a NF to be added to IF, FOF, to a variety of foods as well as to food supplements.

The Panel considers that there are no concerns regarding genotoxicity of the NF.

The Panels considers that a NOAEL could not be established from the 90-day oral toxicity study with the NF. However, the intake of 2'-FL and DFL in breastfed infants on a per kg bw basis is expected to be safe also for other population groups.

The Panel notes that the anticipated daily intake of the NF from the consumption of IF (only), in infants up to 16 weeks of age, does not exceed the high intake level of 2'-FL and DFL in breastfed infants per kg bw. The anticipated daily intake of the NF for the proposed uses at their respective maximum use levels is unlikely to exceed the high intake level of 2'-FL and DFL in breastfed infants per kg bw. Thus, the Panel considers that the intake of the NF for the proposed uses at their respective maximum use levels can be considered safe.

The maximum daily intake of the NF as food supplements (i.e. 4 g/day) for individuals above 1 year of age does not exceed the high intake level of 2'-FL and DFL in breastfed infants per kg bw. Food supplements are not intended to be used if other foods with added NF or 2'-FL (as well as breast milk for young children) are consumed the same day.

For foods for special medical purposes, as the applicant did not propose maximum use levels, the Panel considers that the maximum use levels of the NF should be in accordance with the particular nutritional requirements of the population segment for which the products are intended but in any case not higher than the maximum levels specified for the proposed food uses or the maximum daily intake proposed for food supplements.

5. Conclusions

The Panel concludes that the NF, a mixture of 2'-FL and DFL, is safe under the proposed conditions of use. The target population is the general population, except for food supplements for which the target

population is individuals above 1 year of age. Food supplements are not intended to be used if other foods with added NF or 2'-FL (as well as breast milk for young children) are consumed the same day.

The Panel could not have reached the conclusions on the safety of the NF under the proposed conditions of use without the following data claimed as proprietary by the applicant:

- annexes to the dossier which relate to the identity, the production process, production microorganism, composition and specifications of the NF (see annexes indicated in Section 2.1)
- bacterial reverse mutation test (unpublished study report, 2017a), *in vitro* micronucleus test (unpublished study report, 2017b), and 90-day oral toxicity study with the NF (unpublished study report, 2018) including the summary table of the statistically significant observations in the 90-day study (Appendix B.3 to the dossier). The results of these studies have been published by Phipps et al. (2018).

Steps taken by EFSA

1) Letter from the European Commission to the European Food Safety Authority with the request for a scientific opinion on the safety of 2'-fucosyllactose/difucosyllactose mixture. Ref. Ares(2018)3455328, dated 29/06/2018.
2) On 29/06/2018, a valid application on 2'-fucosyllactose/difucosyllactose mixture, which was submitted by Glycom A/S, was made available to EFSA by the European Commission through the Commission e-submission portal (NF 2018/0401) and the scientific evaluation procedure started.
3) On 17/10/2018 and 10/12/2018, EFSA requested the applicant to provide additional information to accompany the application and the scientific evaluation was suspended.
4) On 29/10/2018 and on 12/03/2019, additional information was provided by the applicant through the Commission e-submission portal and the scientific evaluation was restarted.
5) During its meeting on 15/05/2019, the NDA Panel, having evaluated the data, adopted a scientific opinion on the safety of 2'-fucosyllactose/difucosyllactose mixture as a NF pursuant to Regulation (EU) 2015/2283.

References

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

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

Asakuma S, Urashima T, Akahori M, Obayashi H, Nakamura T, Kimura K, Watanabe Y, Arai I and Sanai Y, 2008. Variation of major neutral oligosaccharides levels in human colostrum. European Journal of Clinical Nutrition, 62, 488–494.

Asakuma S, Hatakeyama E, Urashima T, Yoshida E, Katayama T, Yamamoto K, Kumagai H, Ashida H, Hirose J and Kitaoka M, 2011. Physiology of consumption of human milk oligosaccharides by infant gut-associated *Bifidobacteria*. Journal of Biological Chemistry, 286, 34583–34592[plus supplementary data]

Austin S, De Castro CA, Benet T, Hou Y, Sun H, Thakkar SK, Vinyes-Pares G, Zhang Y and Wang P, 2016. Temporal change of the content of 10 oligosaccharides in the milk of Chinese urban mothers. Nutrients, 8, 346, 22 pp. https://doi.org/10.3390/nu8060346

AVA (Agri-Food & Veterinary Authority of Singapore), 2018. Available online: https://www.ava.gov.sg/docs/default-source/legislation/sale-of-food-act/circular-on-food-(amdt)-regns-2018-rev-23mar2018.pdf

Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B and Shao Y, 1997. The complete genome sequence of *Escherichia coli* K-12. Science, 277, 1453–1462.

Bode L, 2012. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology, 22, 1147–1162.

Brand-Miller JC, McVeagh P, McNeil Y and Gillard B, 1995. Human milk oligosaccharides are not digested and absorbed in the small intestine of young infants. Proceedings of the Nutrition Society of Australia, 19, 44.

Brand-Miller JC, McVeagh P, McNeil Y and Messer M, 1998. Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. Journal of Pediatrics, 133, 95–98.

Chaturvedi P, Warren CD, Buescher CR, Pickering LK and Newburg DS, 2001b. In: Newberg DS (ed). Survival of human milk oligosaccharides in the intestine of infants. In: Bioactive components of human milk. (Advances in experimental medicine and biology, volume 501). Springer Science+Business, Media, New York. pp. 315–323.

Coppa GV, Pierani P, Zampini L, Carloni I, Carlucci A and Gabrielli O, 1999. Oligosaccharides in human milk during different phases of lactation. Acta Paediatrica, suppl, 430, 89–94.

Coppa GV, Pierani P, Zampini L, Bruni S, Carloni I and Gabrielli O, 2001. Characterization of oligosaccharides in milk and faeces of breast-fed infants by high-performance anion-exchange chromatography. Advances in Experimental Medicine and Biology, 501, 307–314.

Coppa GV, Gabrielli O, Zampini L, Galeazzi T, Ficcadenti A, Padella L, Santoro L, Soldi S, Carlucci A, Bertino E and Morelli L, 2011. Oligosaccharides in 4 different milk groups, *Bifidobacteria*, and *Ruminococcus obeum*. Journal of Pediatric Gastroenterology and Nutrition, 53, 80–87.

EFSA (European Food Safety Authority), 2011. Use of the EFSA Comprehensive European Food Consumption Database in Exposure Assessment. EFSA Journal 2011;9(3):2097, 34 pp. https://doi.org/10.2903/j.efsa.2011.2097

EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Girones R, Koutsoumanis K, Lindqvist R, Nørrung B, Robertson L, Ru G, Fernandez Esc amez PS, Sanaa M, Simmons M, Skandamis P, Snary E, Speybroeck N, Ter Kuile B, Threlfall J, Wahlstrom H, Cocconcelli PS, Peixe L, Maradona MP, Querol A, Suarez JE, Sundh I, Vlak J, Barizzone F, Correia S and Herman L, 2018. Statement on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 7: suitability of taxonomic units notified to EFSA until September 2017. EFSA Journal 2018;16(1):5131, 43 pp. https://doi.org/10.2903/j.efsa.2018.5131

EFSA FEEDAP Panel (EFSA Panel on Additives and Products or Substances used in Animal Feed), Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos ML, Bories G, Chesson A, Cocconcelli PS, Flachowsky G, Gropp J, Kolar B, Kouba M, Lopez-Alonso M, Lopez Puente S, Mantovani A, Mayo B, Ramos F, Saarela M, Villa RE, Wallace RJ, Wester P, Glandorf B, Herman L, K€arenlampi S, Aguilera J, Anguita M, Brozzi R and Galobart J, 2018. Guidance on the characterisation of microorganisms used as feed additives or as production organisms. EFSA Journal 2018;16(3):5206, 24 pp. https://doi.org/10.2903/j.efsa.2018.5206

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2013. Scientific Opinion on nutrient requirements and dietary intakes of infants and young children in the European Union. EFSA Journal 2013;11 (10):3408, 103 pp. https://doi.org/10.2903/j.efsa.2013.3408

EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific Opinion on the essential composition of infant and follow-on formulae. EFSA Journal 2014;12(7):3760, 106 pp. https://doi.org/10.2903/j.efsa.2014.3760

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

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

EFSA Scientific Committee, 2017. Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (Question no EFSA-Q-2016-00489, adopted: 26 April 201). EFSA Journal 2017;15(5):4849, 58 pp. https://doi.org/10.2903/j.efsa.2017.4849. Available online: https://www.efsa.europa.eu/en/efsajournal/pub/4849

Elison E, Vigsnaes LK, Rindom Krogsgaard L, Rasmussen J, Sørensen N, McConnell B, Hennet T, Sommer MO and Bytzer P, 2016. Oral supplementation of healthy adults with 2'-*O*-fucosyllactose and lacto-*N*-neotetraose is well tolerated and shifts the intestinal microbiota. British Journal of Nutrition, 116, 1356–1368 [plus supplementary table].

EMA (European Medicines Agency), 2010. Guideline on Repeated Dose Toxicity. European Agency for the Evaluation of Medicinal Products. Committee for Human Medicinal Products (CHMP), London, UK CPMP/SWP/1042/99 Rev 1 Corr.

Engfer MB, Stahl B, Finke B, Sawatzki G and Daniel H, 2000. Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. American Journal of Clinical Nutrition, 71, 1589–1596.

Erney RM, Malone WT, Skelding MB, Marcon AA, Kleman-Leyer KM, O'Ryan ML, Ruiz-Palacios G, Hilty MD, Pickering LK and Prieto PA, 2000. Variability of human milk neutral oligosaccharides in a diverse population. Journal of Pediatric Gastroenterology and Nutrition, 30, 181–192.

Erney R, Hilty M, Pickering L, Ruiz-Palacios G and Prieto P, 2001. Human milk oligosaccharides: a novel method provides insight into human genetics. In: Bioactive components of human milk. 8th International Conference of the International Society for Research on Human Milk and Lactation, Oct. 25-29, 1997, Plymouth, Mass. Ed., Newburg DS. Kluwer Academic/Plenum Publishers, New York. Advances in Experimental Medicine and Biology, vol 501, 285–297.

FARRP (Food Allergy Research and Resource Program), 2017. AllergenOnline version 17: home of the FARRP allergen protein database. University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP), Lincoln, NE. Available online: http://www.allergenonline.org/ [Accessed: 18 January 2017]

FDA (Food and Drug Administration), 2015. GRAS (Generally Recognized as Safe) for 2'-O-fucosyllactose. GNR 000571. Available online: https://www.accessdata.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=571

FDA (Food and Drug Administration), 2016. GRAS (Generally Recognized as Safe) for 2'-O-fucosyllactose. GNR 000650. Available online: https://www.fda.gov/downloads/food/ingredientspackaginglabeling/gras/noticeinventory/ucm513832.pdf

Galeotti F, Coppa GV, Zampini L, Maccari F, Galeazzi T, Padella L, Santoro L, Gabrielli O and Volpi N, 2012. On-line high-performance liquid chromatography-fluorescence detection-electrospray ionization-mass spectrometry profiling of human milk oligosaccharides derivatized with 2-aminoacridone. Analytical Biochemistry, 430, 97–104 [plus supplementary Appendix A].

Galeotti F, Coppa GV, Zampini L, Maccari F, Galeazzi T, Padella L, Santoro L, Gabrielli O and Volpi N, 2014. Capillary electrophoresis separation of human milk neutral and acidic oligosaccharides derivatized with 2-aminoacridone. Electrophoresis, 35, 811–818.

Gnoth MJ, Kunz C, Kinne-Saffran E and Rudloff S, 2000. Human milk oligosaccharides are minimally digested in vitro. Journal of Nutrition, 130, 3014–3020.

Gorbach SL, 1978. Recombinant DNA: an infectious disease perspective. Journal of Infectious Diseases, 137, 615–623.

Kajzer J, Oliver J and Marriage B, 2016. Gastrointestinal tolerance of formula supplemented with oligosaccharides. FASEB Journal, 30, suppl, abstract 671.4. Available online: http://www.fasebj.org/content/30/1_Supplement/671.4?relatedurls=yes&legid=fasebj;30/1_Supplement/671.4.

Lukjancenko O, Wassenaar TM and Ussery DW, 2010. Comparison of 61 sequenced Escherichia coli genomes. Microbial Ecology, 60, 708–720.

Marriage BJ, Buck RH, Goehring KC, Oliver JS and Williams JA, 2015. Infants fed a lower calorie formula with 2'-fucosyllactose (2'FL) show growth and 2'FL uptake like breast-fed infants. Journal of Pediatric Gastroenterology and Nutrition, 61, 649–658.

McGuire MK, Meehan CL, McGuire MA, Williams JE, Foster J, Sellen DW, Kamau-Mbuthia EW, Kamundia EW, Mbugua S, Moore SE, Prentice AM, Kvist LJ, Otoo GE, Brooker SL, Price WJ, Shafii B, Placek C, Lackey KA, Robertson B, Manzano S, Ruíz L, Rodríguez JM, Pareja RG and Bode L, 2017. What's normal? Oligosaccharide concentrations and profiles in milk produced by healthy women vary geographically. The American Journal of Clinical Nutrition, 105, 1086–1100 [plus supplementary data].

Morrow AL, Ruiz-Palacios GM, Altaye M, Jiang X, Guerrero ML, Meinzen-Derr JK, Farkas T, Chaturvedi P, Pickering LK and Newburg DS, 2004. Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants. Journal of Pediatrics, 145, 297–303.

Muhldorfer I and Hacker J, 1994. Genetic aspects of *Escherichia coli* virulence. Microbial Pathogenesis, 16, 171–181.

Musumeci M, Simpore J, D'Agata A, Sotgiu S and Musumeci S, 2006. Oligosaccharides in colostrum of Italian and Burkinabe women. Journal of Pediatric Gastroenterology and Nutrition, 43, 372–378.

OECD (Organisation for Economic Co-operation and Development), 1986. Recombinant DNA safety considerations ["Blue Book"]. Available online: https://www.oecd.org/sti/biotech/40986855.pdf

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

OECD (Organisation for Economic Co-operation and Development), 1998a. Test No. 408: Repeated dose 90-day oral toxicity study in rodents. In: OECD guidelines for the testing of chemicals, Section 4: Health effects, 10 pp.

OECD (Organisation for Economic Co-operation and Development), 1998b. OECD Principles of good laboratory practice (as revised in 1997). OECD series on principles of good laboratory practice and compliance monitoring, number 1, ENV/MC/CHEM(98)17, 41 pp.

OECD (Organisation for Economic Co-operation and Development), 2014. Test No. 487: In vitro mammalian cell micronucleus test. In: OECD guidelines for the testing of chemicals, Section 4: Health effects, 23 pp.

Oftedal OT, Nicol SC, Davies NW, Sekii N, Taugik E, Fukuda K, Saito T and Urashima T, 2014. Can an ancestral condition for milk of oligosaccharides be determined? Evidence from the Tasmanian echidna (*Tachyglossus aculeatus setosus*). Glycobiology, 24, 826–839.

Phipps KR, Baldwin N, Lynch B, Flaxmer J, Soltesova A, Gilby B, Miks MH and Rohrig CH, 2018. Safety evaluation of a mixture of the human-identical milk oligosaccharides 2'-fucosyllactose and difucosyllactose. Food and Chemical Toxicology, 120, 552–565.

Pratico G, Capuani G, Tomassini A, Baldassarre ME, Delfini M and Miccheli A, 2014. Exploring human breast milk composition by NMR-based metabolomics. Natural Product Research, 28, 95–101.

Puccio G, Alliet P, Cajozzo C, Janssens E, Corsello G, Sprenger N, Wernimont S, Egli D, Gosoniu L and Steenhout P, 2017. Effects of infant formula with human milk oligosaccharides on growth and morbidity: a randomized multicenter trial. Journal of Pediatric Gastroenterology and Nutrition, 64, 624–631.

Rudloff S and Kunz C, 2012. Milk oligosaccharides and metabolism in infants. Advances in Nutrition, 3, 398S–405S.

Spevacek AR, Smilowitz JT, Chin EL, Underwood MA, German JB and Slupsky CM, 2015. Infant maturity at birth reveals minor differences in the maternal milk metabolome in the first month of lactation. Journal of Nutrition, 145, 1698–1708[plus supplemental tables].

Thurl S, Munzert M, Henker J, Boehm G, Muller-Werner B, Jelinek J and Stahl B, 2010. Variation of human milk oligosaccharides in relation to milk groups and lactational periods. British Journal of Nutrition, 104, 1261–1271.

Thurl S, Munzert M, Boehm G, Matthews C and Stahl B, 2017. Systematic review of the concentrations of oligosaccharides in human milk. Nutrition Reviews, 75, 920–933.

Unpublished study report, 2015a. An in vitro micronucleus assay with 2'FL in cultured peripheral human lymphocytes., Project, 507433.

Unpublished study report, 2015b. An in vitro micronucleus assay with 2'-O-fucosyllactose in cultured peripheral human lymphocytes., Project, 507398.

Unpublished study report, 2015c. Evaluation of the mutagenic activity of 2'FL in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (plate incorporation and pre-incubation methods). Project 507432.

Unpublished study report, 2015d. 2'-FL – 13-week oral (gavage) juvenile toxicity study in the rat followed by a 4-week treatment-free period. Study Number, AB20757.
Unpublished study report, 2017a. 2'-O-Fucosyllactose/Difucosyllactose mixture: Bacterial Reverse Mutation Test. Study Number, DW08NF.
Unpublished study report, 2017b. 2'-O-Fucosyllactose/Difucosyllactose mixture. In Vitro Micronucleus Test in Human Lymphocytes. Study Number, RM71CK.
Unpublished study report, 2017c. 2'-O-Fucosyllactose and Difucosyllactose (2'-FL/DFL): 14-Day Dose Range Finding Study in the Neonatal Crl:CD(SD) Rat by Oral (Gavage)Administration. Study Number: RW47RS.
Unpublished study report, 2018. 2'-O-Fucosyllactose and Difucosyllactose (2'-FL/DFL): 90-Day Toxicity Study in the Neonatal Crl:CD(SD) Rat by Oral (Gavage) Administration Followed by a 4-Week Recovery Period. Study Number: YJ14GH.
Urashima T, Taufik E, Fukuda K and Asakuma S, 2013. Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. Bioscience, Biotechnology, and Biochemistry, 77, 455–466.
Urashima T, Yamaguchi E, Ohshima T, Fukuda K and Saito T, 2018. Chemical structures of oligosaccharides in milk of the raccoon (*Procyon lotor*). Glycoconjugate Journal, 35, 275–286.
U.S. EPA (U.S. Environmental Protection Agency), 1997. *Escherichia coli* K-12 final risk assessment: attachment I–final risk assessment of Escherichia coli K-12 derivatives. Available online: http://www2.epa.gov/sites/production/files/2015-09/documents/fra004.pdf [Accessed: 27 September 2012].

Abbreviations

2'-FL	2'-fucosyllactose
ADME	absorption, distribution, metabolism and excretion
AST	aspartate aminotransferase
bw	body weight
CFU	colony forming units
DFL	difucosyllactose
DSMZ	German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen)
EU	endotoxin units
FAIM	Food Additive Intake Model
FOF	follow-on formula
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
HACCP	Hazard Analysis Critical Control Points
HMO	human milk oligosaccharide
HPAEC/PAD	high performance anion exchange chromatography/pulsed amperometric detection
IF	infant formula
LOR	limit of reporting
LUC	large unstained cells
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
MDO	membrane-derived oligosaccharide
MS	mass spectrometry
NF	novel food
NOAEL	no observed adverse effect level
NOESY	Nuclear Overhauser Effect SpectroscopY
NMR	^{1}H-nuclear magnetic resonance spectroscopy
OECD	Organisation for Economic Co-operation and Development
Ph	European Pharmacopoeia
QPS	qualified presumption of safety
RDW	red cell distribution width
RH	residual humidity
RT	retention time
UHT	ultra-high temperature
UV	ultraviolet

Appendix A – Summary results of the 90-day repeated dose toxicity study with the NF

Summary results of parameters with statistically significant differences in the 90-day repeated dose toxicity study with the NF (Phipps et al., 2018; Unpublished study report, 2018)

Parameters	Exposure (day)	Sex	Control	Low dose (1,000 mg/kg bw)	Mid dose (3,000 mg/kg bw)	High dose (5,000 mg/kg bw)	Oligofructose control (5,000 mg/kg bw)
Body weight (g)	9	F	31.2 ± 3.26	32.2 ± 2.02	32.6 ± 1.09	33.5 ± 2.52*	30.8 ± 2.70
Ulna length (mm)	64	M	39.5 ± 0.83	38.3 ± 0.94**	38.6 ± 0.44	39.5 ± 1.04	39.1 ± 1.20
	64	F	36.7 ± 0.90	35.7 ± 0.46*	35.6 ± 0.50*	36.3 ± 1.13*	36.4 ± 1.21
Arena observations							
Activity count	90	M	17.5 ± 5.0	12.6 ± 5.8	13.2 ± 6.6	17.4 ± 5.6	12.3 ± 6.0^
		F	23.8 ± 4.5	25.4 ± 6.0	25.0 ± 6.1	19.8 ± 5.1*	20.6 ± 4.3
Sexual maturation							
Balano preputial separation (day of age)	90	M	43.7 ± 1.91	44.4 ± 1.17	45.3 ± 0.82	45.4 ± 2.23*	45.4 ± 2.59^
Haematology							
Haematocrit (L/L)	90	M	0.39 ± 0.01	0.45 ± 0.02**	0.45 ± 0.01**	0.43 ± 0.04**	0.39 ± 0.01
		F	0.38 ± 0.02	0.44 ± 0.02**	0.44 ± 0.02**	0.39 ± 0.04	0.38 ± 0.01
Haemoglobin (g/dL)	90	M	14.8 ± 0.35	15.4 ± 0.54*	15.2 ± 0.37*	15.3 ± 0.78*	14.8 ± 0.35
Erythrocytes ($\times 10^{12}$/L)	90	M	7.46 ± 0.33	8.32 ± 0.31**	8.04 ± 0.46**	8.04 ± 0.67**	7.36 ± 0.20
		F	7.06 ± 0.34	7.80 ± 0.33**	7.83 ± 0.36**	7.08 ± 0.71	6.95 ± 0.27
MCH (pg)	90	M	19.8 ± 0.62	18.5 ± 0.55*	19.0 ± 0.93*	19.1 ± 1.21*	20.1 ± 0.48
		F	20.8 ± 0.68	19.2 ± 0.78**	19.0 ± 0.70**	20.3 ± 1.34	21.3 ± 0.62
MCHC (g/dL)	90	M	37.6 ± 0.57	33.9 ± 0.63**	33.6 ± 0.51**	35.9 ± 2.50**	37.9 ± 0.4
		F	38.6 ± 0.85	33.7 ± 0.86**	33.6 ± 0.95**	36.8 ± 2.85	39.3 ± 0.70
MCV	90	M	52.7 ± 1.46	54.5 ± 1.03*	56.5 ± 2.30**	53.3 ± 1.72	52.9 ± 0.9
		F	53.9 ± 1.22	56.9 ± 1.38**	56.6 ± 1.41**	55.2 ± 0.90	54.1 ± 1.1
Red cell distribution width (%)	90	M	12.4 ± 0.59	11.7 ± 0.32*	11.6 ± 0.50*	12.3 ± 0.83	12.7 ± 0.40
Leucocytes ($\times 10^9$/L)	90	F	10.29 ± 3.86	6.92 ± 0.93*	6.34 ± 1.37**	10.82 ± 5.44	10.64 ± 2.08
Lymphocytes ($\times 10^9$/L)	90	F	8.80 ± 3.65	5.75 ± 0.87**	5.15 ± 1.14**	7.67 ± 1.23	8.80 ± 2.03
Eosinophils ($\times 10^9$/L)	90	M	0.16 ± 0.06	0.11 ± 0.04	0.09 ± 0.02*	0.18 ± 0.11	0.17 ± 0.06
		F	0.14 ± 0.04	0.08 ± 0.03*	0.09 ± 0.04*	0.09 ± 0.03*	0.11 ± 0.04
Basophils ($\times 10^9$/L)	90	M	0.04 ± 0.01	0.08 ± 0.03**	0.08 ± 0.03**	0.07 ± 0.04**	0.06 ± 0.03^
		F	0.03 ± 0.02	0.04 ± 0.02**	0.05 ± 0.02**	0.04 ± 0.02**	0.03 ± 0.01

Parameters	Exposure (day)	Sex	Control	Low dose (1,000 mg/kg bw)	Mid dose (3,000 mg/kg bw)	High dose (5,000 mg/kg bw)	Oligofructose control (5,000 mg/kg bw)
Monocytes ($\times 10^9$/L)	90	F	0.22 ± 0.05	0.15 ± 0.03**	0.12 ± 0.04**	0.17 ± 0.08**	0.24 ± 0.08
LUC ($\times 10^9$/L)	90	M	0.03 ± 0.01	0.06 ± 0.01*	0.06 ± 0.02**	0.06 ± 0.03**	0.05 ± 0.02
Platelet count ($\times 10^9$/L)	90	F	976 ± 105.2	907 ± 69.7	813 ± 116.6*	881 ± 137.9*	872 ± 113.6^
Prothrombin time (sec)	90	M	20.6 ± 1.5	23.1 ± 2.3*	23.6 ± 3.22*	22.2 ± 2.76*	22.9 ± 2.22^
	End 4-week recovery period	M	24.0 ± 40.8	n.a.	n.a.	23.2 ± 3.26	22.6 ± 3.33
Clinical chemistry parameters							
AST (U/L)	90	M	74 ± 7.3	85 ± 11.8*	84 ± 7.6*	75 ± 4.8	78 ± 10
		F	70 ± 11.6	86 ± 13.2*	82 ± 13.7	70 ± 11.8	69 ± 7.6
Albumin (g/L)	90	M	34 ± 1.1	33 ± 1.2	32 ± 1.7**	35 ± 1.8	35 ± 1.2
	90	F	40 ± 1.8	37 ± 2.7*	37 ± 1.5*	39 ± 3.8*	38 ± 1.7
Urea (mmol/L)	90	F	5.69 ± 0.58	6.77 ± 0.76*	6.86 ± 1.16*	5.80 ± 1.09	5.94 ± 0.48
Creatinine (μmol/L)	90	M	27 ± 2.5	25 ± 3.1	25 ± 3.1	23 ± 2.1**	25 ± 2.5
Sodium (mmol/L)	90	M	144 ± 1.3	142 ±1.2	143 ± 1.2	143 ± 1.3	142 ± 1.0^^
		F	142 ± 0.5	142 ± 1.6	141 ± 1.4	141 ± 1.2**	141 ± 1.0^
Chloride (mmol/L)	90	M	100 ± 0.8	99 ± 1.3	100 ± 1.0	99 ± 1.2*	98 ± 1.5^^
Calcium (mmol/L)	90	M	2.39 ± 0.06	2.42 ± 0.07	2.40 ± 0.06	2.45 ± 0.06	2.44 ± 0.07^
		F	2.43 ± 0.08	2.51 ± 0.10	2.43 ± 0.05	2.54 ± 0.08**	2.41 ± 0.07
Inorganic phosphorus (mmol/L)	90	F	1.77 ± 0.23	1.83 ± 0.31	1.80 ± 0.21	2.03 ± 0.24**	1.77 ± 0.24
Urinalysis							
pH	90	F	6.7 ± 0.4	6.8 ± 0.3	7.1 ± 0.5	7.4 ± 0.9**	6.8 ± 0.4
Total creatinine (μmol)	90	F	45.35 ± 6.69	42.45 ± 5.63	41.25 ± 8.27	36.08 ± 9.78*	42.45 ± 8.19
Organ weight							–
Kidneys relative	90	M	3.332	3.674**	3.387	3.393	3.153
Seminal vesicles relative	90	M	1.745	1.991*	1.893	1.660	1.673
Thymus relative	90	M	0.321	0.400*	0.387*	0.399*	0.331

NF: novel food; bw: body weight; AST: aspartate aminotransferase; LUC: large unstained cells; MCH: mean cell haemoglobin; MCV: mean cell volume; MCHC: mean cell haemoglobin concentration; RDW: red cell distribution width.

*: Significantly different from control ($p < 0.05$) (Vehicle control vs 2'-FL/DFL mixture-treated groups).

**: Significantly different from control ($p < 0.01$) (control vs 2'-FL/DFL mixture-treated groups).

^: Significantly different from control ($p < 0.05$) (control vs reference control).

^^: Significantly different from control ($p < 0.01$) (control vs reference control).