Application for the Approval of 2'-*O*-Fucosyllactose (2'-FL) and Lacto-*N*-neotetraose (LNnT) Under Standard 1.5.1 (Novel Foods) of the Australia and New Zealand Food Standards Code

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Application for the Approval of 2'-O-Fucosyllactose (2'-FL) and Lacto-N-neotetraose (LNnT) Under Standard 1.5.1 (Novel Foods) of the Australia and New Zealand Food Standards Code

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INTRODUCTION

Glycom A/S (Glycom) is a Danish food ingredient manufacturer that has developed the technology to manufacture human-identical milk oligosaccharides (HiMOs)[1]. Initially, Glycom developed chemical-synthetic process technology before moving to fermentation technology, as a more scalable and cost-effective route that would provide the same benefit at lowest cost to the consumer. These HiMOs are highly purified; they are chemically identical to those in mother's milk, and will be added at levels within the ranges naturally found.

The first 2 HiMOs to be commercialised by Glycom are 2'-*O*-fucosyllactose (2'-FL) and lacto-*N*-neotetraose (LNnT). Both of these ingredients (obtained by either chemical synthesis and fermentation processes) have gained approval and notification in the European Union as novel food ingredients for use in a variety of food products including infant formulae, follow-on formulae, processed cereal-based food and baby food for infants and young children, milk-based drinks and similar products intended for young children, dietary foods for special medical purposes and meal replacements as well as a range of other food groups for the general population. They have also successfully achieved Generally Recognized as Safe (GRAS) status for these same food uses in the United States (U.S.), which has been notified to the Food and Drug Administration (FDA) without questions. Just recently, 2'-FL and LNnT have been granted approval for use in infant formula and growing up milks in Singapore, and 2'-FL has been authorised for use in infant formula in Israel.

This application is for the approval of 2'-FL and LNnT as novel foods in Australia/New Zealand. 2'-FL is proposed for addition at levels of up to 1.2 g/L, either alone or in combination with up to 0.6 g/L of LNnT, in infant formula, follow-on formula, and formulated supplementary foods for young children. The approval is sought for 2'-FL and LNnT produced by fermentation, by two similar but independent processes. As stated above, the final ingredients are highly-purified; they are produced using living fermentation organisms, but secreted from the organism which are removed completely, and the ingredients are subjected to a number of purification steps (similar to the production of vitamins). The 2'-FL and LNnT ingredients will not be manufactured within Australia/New Zealand; only the finished ingredients, or food products containing it, will be imported. Thus, no processing aids (including the fermentation organism) will enter the territory of Australia/New Zealand. This application is intended to demonstrate:

1. 2'-FL and LNnT are chemically identical to those found in human breast milk.

2. 2'-FL and LNnT will be added at levels not exceeding those found in human breast milk.

[1] Throughout this application, the term "human-identical milk oligosaccharides (HiMOs)" is used to refer to the manufactured forms of these substances (*e.g.*, 2'-FL and LNnT obtained by either chemical synthesis or microbial fermentation), while the term "human milk oligosaccharides (HMOs)" is used to refer to their naturally occuring counterparts in human breastmilk. Glycom has demonstrated that the HiMOs that are the subject of the current application (2'-FL and LNnT manufactured by fermentation) are identical to the 2'-FL and LNnT that are present naturally in human breastmilk.

3. 2’-FL and LNnT are highly purified.

4. 2’-FL, either alone or in combination with LNnT, are well tolerated and do not pose any safety concerns, based on their history of consumption through breastmilk, as well as numerous safety studies (both preclinical and clinical) that have been conducted.

5. 2’-FL and LNnT are non-digestible oligosaccharides that will provide functional benefits.

This dossier has been prepared in accordance with the Food Standards Australia New Zealand (FSANZ) *Application Handbook,* specifically:

- Chapter 3.1 – General Requirements for Applications (all sections)
- Guideline 3.5.2 – Novel Food (all sections)
- Guideline 3.6.2 – Special Purpose Foods – Infant Formula Product (all sections)
- Guideline 3.6.3 – Special Purpose Foods – Other Foods (all sections)
- Guideline 3.3.2 – Processing Aids (sections D and E)

GENERAL REQUIREMENTS

This section is completed in accordance with Chapter 3.1 (General Requirements for Applications) of the Food Standards Australia New Zealand Application Handbook (FSANZ, 2016).

1. Applicant Details

Contact Information

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Attention of:

Nature of Applicant's Business

Glycom is a privately held company founded in 2005, specialising in the development, synthesis, and commercialisation of human-identical milk oligosaccharides. Consisting of a R&D headquarters and a production facility, it synergistically covers the whole value chain from research, intellectual property protection (IP), development, over technology transfer to engineering and production, isolation-purification, product release and product approval. The company comprises of ca. 120 employees, approximately half of which are scientists specialising in compliance, quality, technical engineering, carbohydrate chemistry, biochemistry and analytics, enzymology, strain development, microbial fermentation, process isolation-purification, regulatory and IP.

Details of Other Parties Involved with the Application

The following parties are involved in the preparation, submission and stewardship of this application:

Intertek Scientific & Regulatory Consultancy
Room 1036, Building A8
Cody Technology Park
Ively Road
Farnborough, UK
GU14 0LX

2. Purpose of the Application

Human breast milk contains a number of structurally diverse oligosaccharides, termed human milk oligosaccharides (HMOs)[2], which include 2'-*O*-fucosyllactose (2'-FL) and lacto-*N*-neotetraose (LNnT). Glycom (A/S) (Glycom) has developed means to manufacture 2'-FL and LNnT by microbial fermentation. The 2'-FL and LNnT produced by microbial fermentation are chemically and structurally identical to the oligosaccharides that are naturally present in human breast milk. Glycom intends to use 2'-FL (either alone or in combination with LNnT) in infant formula, follow-on formula, and formulated supplementary foods for young children, specifically milk products, that are intended for use by young children aged 1 to 3 years.

It is Glycom's view that 2'-FL and LNnT meet the definition of a "*non-traditional food*", as defined in Standard 1.5.1, on the basis that they are considered to be a "*substance, where that substance, or the source from which it is derived, does not have a history of human consumption as a food in Australia or New Zealand*". Furthermore, given that 2'-FL and LNnT produced by fermentation do not have a history of human consumption as a food in Australia or New Zealand (even though their identical counterparts are naturally present in human milk), they are considered to be a novel food requiring an assessment of public health and safety consideration. As such, 2'-FL and LNnT obtained by microbial fermentation are considered to be within the scope of the definition of novel food for the purposes of Standard 1.5.1 of the Australia New Zealand Food Standards Code (hereafter referred to as the Code) (FSANZ, 2016). The purpose of the application herein is to amend Schedule 25 (Permitted Novel Foods) of the Code to include 2'-FL and LNnT obtained by microbial fermentation in the table to section S25-2. Furthermore, it is recognised that the approval of 2'-FL and LNnT as a novel food may require consideration and possible changes to the following Standards and their associated Schedules as relevant:

- Standard 2.9.1: Infant Formula Products
- Standard 2.9.3, Division 4: Formulated Supplementary Foods for Young Children
- Schedule 3: Identity and Purity

The information presented in this application support the safe and suitable use of 2'-FL and LNnT for their proposed food applications.

3. Justification for the Application

3.1 Regulatory Impact Information

3.1.1 Costs and Benefits of Application

a) Impact on the Consumer

The proposed uses of 2'-FL and LNnT is consistent with efforts to produce products that better match the nutrient composition of human milk, as set forth by principles in the Australia and New Zealand Food Regulation Ministerial Council's Policy Guideline on the *Regulation of Infant Formula Products* and the Codex *Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants* (Codex Stan 72). Consumers will benefit from the availability of infant formula products on the market that better reflects the oligosaccharide composition in human breast milk. The abundance and unique presence of HMOs in human milk suggest they must play an important physiological role for the

[2] Throughout this application, the term "human-identical milk oligosaccharides (HiMOs)" is used to refer to the manufactured forms of these substances (*e.g.*, 2'-FL and LNnT obtained by either chemical synthesis or microbial fermentation), while the term human milk oligosaccharides (HMOs)" is used to refer to their naturally occuring counterparts in human breastmilk. Glycom has demonstrated that the HiMOs that are the subject of the current application (2'-FL and LNnT manufactured by fermentation) are identical to the 2'-FL and LNnT that are present naturally in human breastmilk.

developing infant, as supported by various studies (preclinical and clinical) that have examined their effects on human health. An in-depth discussion of the purpose for adding Glycom's HiMOs to infant formula products and formulated supplementary foods for young children, as well as their associated beneficial health effects, is presented in Section E of this application.

The introduction of Glycom's 2'-FL and LNnT as novel ingredients in infant formula and follow-on formula, as well as formulated supplementary foods for young children, is not expected to have any negative impact on the consumers. The safety of the 2'-FL and LNnT ingredients has been well demonstrated, and they have been approved for similar purposes in other jurisdictions worldwide, including the EU, U.S., Israel, and Singapore (see Sections C.7 and D.5).

b) Impact on the Industry

The approval of 2'-FL and LNnT as novel foods in Australia/New Zealand will allow for the development of formula products that better reflect the composition of human breast milk. This will foster innovation in the industry and promote continued research into the importance of optional ingredients that are added to such products. The approval of 2'-FL and LNnT as new optional ingredients that are permitted for addition to infant formula, follow-on formula, and formulated supplementary foods for young children will also facilitate differentiation and competition in the market.

c) Impact on the Government

There is no apparent impact on government agencies by the controlled addition of 2'-FL (either alone or in combination with LNnT) as an ingredient in foods. The authorisation of 2'-FL and LNnT as novel food ingredients will be an extension to the range of other non-digestible oligosaccharides (inulin-type fructans and galacto-oligosaccharides) already permitted for inclusion in infant formula, follow-on formula, and formulated supplementary foods for young children. It is notable however that a high number of key studies on milk oligosaccharides were performed by researchers being supported by Australian government funds (*e.g.*, Dr. Patricia McVeagh, Dr. Janette C. Brand-Miller, Dr. Michael Messer, Dr. Bing Wang). Therefore, the approval of 2'-FL and LNnT by FSANZ would appreciate and link these fundamental research endeavours with actual application to human nutrition and allow for easier access to this HiMO for research purposes, and foster employment by promoting scientific research within Australia/New Zealand.

3.2 Impact on International Trade

Glycom's 2'-FL and LNnT have already obtained market authorisation as ingredients in a wide range of food products in other jurisdictions. The impact on international trade is anticipated to be the following:

- Approval of 2'-FL and LNnT as novel foods will, in the future, promote international trade and reduction of technical barriers to trade, while continuing to protect public health and safety.
- Manufacturers and importers in Australia/New Zealand wishing to sell 2'-FL and LNnT, or foods containing these ingredients, would benefit.
- The global opportunities for the development and sale of 2'-FL and LNnT, or foods containing these ingredients, would be expanded.
- Approval of 2'-FL and LNnT as novel foods gives the domestic industry the chance to provide consumers with products sold in Australia/New Zealand that contain these ingredients.

4. Information to Support the Application

Information is provided in this application to enable the objectives specified in Section 18 of the Food Standards Australia New Zealand (FSANZ) Act to be addressed as follows:

(a) The protection of public health and safety: Information to support objective (a) is provided in Section C of the application, in which the safety of 2'-FL and LNnT, based on the available pre-clinical and human safety data, is discussed in detail.

(b) The provision of adequate information relating to food to enable consumers to make informed choices: Data to support objective (b) are provided in Section B, in which the full compositional information and nutrient profiles of foods containing 2'-FL and LNnT are described in detail. A discussion of the nutritional and health impact of 2'-FL and LNnT as novel food ingredients is also provided in Section E.

(c) The prevention of misleading or deceptive conduct: Information supporting objective (c) is provided in Section F, in which the consumer awareness and potential behaviour in response to 2'-FL and LNnT, and foods containing these ingredients, are described in detail.

This application is prepared in accordance with the relevant sections within the Food Standards Australia New Zealand *Application Handbook* (from 1 March 2016), including the following:

- Chapter 3.1 – General Requirements for Applications (all sections)
- Guideline 3.5.2 – Novel Food (all sections)
- Guideline 3.6.2 – Special Purpose Food – Infant Formula Product (all sections)
- Guideline 3.6.3 – Special Purpose Foods – Other Foods (all sections)
- Guideline 3.3.2 – Processing Aids (sections D and E)

5. Assessment Procedure

Glycom considers the General Procedure (Subdivision D), Cost Category Level 2 (up to 650 hours), to be the most appropriate assessment procedure for the application of 2'-FL (either alone or in combination with LNnT) as a novel food. The safety of the proposed uses of the 2'-FL and LNnT ingredients is largely based on the fact that the use levels remain well within the ranges that occur naturally within human breastmilk. Safety is further corroborated by preclinical and clinical studies conducted with 2'-FL and LNnT (from both fermentation and chemical synthetic processes). These studies have been reviewed by authoritative bodies in the EU, and the U.S. FDA has issued a "no questions" response to the GRAS notices submitted for these ingredients (see Section C.7). A listing of the available preclinical and clinical studies that have been conducted for 2'-FL and LNnT is summarised in Table 5-1 and Table 5-2 below, respectively.

Table 5-1 List of the Available Preclinical Studies Conducted for 2'-FL and LNnT[a]

Information to Support Safety	Assay	Reference	Submitted for Glycom's EU Novel Food Application?	Submitted for Glycom's U.S. FDA GRAS Notice?
Dataset for 2'-FL				
Mutagenicity/ genotoxicity	Ames assay	Verspeek-Rip, 2015	Yes (FSAI)	Yes (GRN 650)
	in vitro micronucleus assay	Verbaan, 2015a	Yes (FSAI)	Yes (GRN 650)
	Ames assay, mouse lymphoma assay	Coulet *et al.*, 2014	Yes (EFSA)	Yes (GRNs 546, 650)
	in vitro micronucleus assay	Verbaan, 2015b	Yes (EFSA)	Yes (GRN 650)
	Ames test, *in vivo* micronucleus assay	Jennewein Biotechnologie GmbH, 2015	No	Yes (GRN 650)
90-day oral toxicity study	OECD No. 408 adapted to begin in neonatal rats	Penard, 2015	Yes (FSAI)	Yes (GRN 650)
	OECD No. 408 adapted to begin in neonatal rats	Coulet *et al.*, 2014	Yes (EFSA)	Yes (GRNs 546, 650)
	OECD No. 408	Jennewein Biotechnologie GmbH, 2015	No	Yes (GRNs 650)
3-week neonatal piglet feeding study	GLP	Hanlon and Thorsrud, 2014	No	Yes (GRNs 650)
Dataset for LNnT				
Mutagenicity/ genotoxicity	Ames assay	Verspeek-Rip, 2016	Yes (FSAI)	Yes (GRN 659)
	in vitro micronucleus assay	Verbaan, 2016	Yes (FSAI)	Yes (GRN 659)
	Ames assay, mouse lymphoma assay	Coulet *et al.*, 2013	Yes (EFSA)	Yes (GRNs 547, 659)
	in vitro micronucleus assay	Verbaan, 2015c	Yes (EFSA)	No
	Ames assay	Prieto, 2005	Yes (EFSA)	Yes (GRNs 547, 659)
28-day oral toxicity study	Gavage study, feeding study (only limited details available; duration of feeding study is not entirely clear)	Prieto, 2005	Yes (EFSA)	Yes (GRNs 547, 659)
	OECD test guideline 407 adapted to begin in neonatal rats	Coulet *et al.*, 2013	Yes (EFSA)	Yes (GRNs 547, 659)
90-day oral toxicity study	OECD No. 408 adapted to begin in neonatal rats	Penard, 2016	Yes (FSAI)	Yes (GRN 659)
	OECD No. 408 adapted to begin in neonatal rats	Coulet *et al.*, 2013	Yes (EFSA)	Yes (GRNs 547, 659)

2'-FL = 2'-O-fucosyllactose; EFSA = European Food Safety Authority; FSAI = Food Safety Authority of Ireland; GLP = Good Laboratory Practice; LNnT = lacto-N-neotetraose; OECD = Organization for Economic Cooperation and Development

[a] Details of these studies are provided in Section C.

Table 5-2 List of the Available Clinical Studies Conducted for 2'-FL and LNnT[a]

Information to Support Safety	Study Groups and Duration of Intervention	Reference	Submitted for Glycom's EU Novel Food Application?	Submitted for Glycom's U.S. FDA GRAS Notice?
Infant feeding studies	• Formula containing **2'-FL** (1.0 to 1.2 g/L) with **LNnT** (0.5 to 0.6 g/L) • Control formula • Breastfed infants • 6 months	Puccio *et al.*, 2017	Yes (reviewed by EFSA)	Yes (GRNs 546, 650)
	• Formula containing **2'-FL** (0.2 g/L) with GOS (2.2 g/L) • Formula containing **2'-FL** (1.0 g/L) with GOS (1.4 g/L) • Control formula • Breastfed infants • 4 months	Marriage *et al.*, 2015	No	Yes (GRN 650)
	• Formula containing **2'-FL** (0.2 g/L) with scFOS (2 g/L) • Control formula • Breastfed infants • 27 to 35 days	Kajzer *et al.*, 2016	No	No
	• Formula containing **LNnT** (0.22 g/L) • Control formula • 6 weeks	Prieto, 2005	Yes (reviewed by EFSA)	Yes (GRNs 547, 659)
Tolerability study in adults	• **2'-FL** alone (up to 20 g/day), **LNnT** alone (up to 20 g/day), or their combination (2:1 ratio for 20 g/day total) • 14 days	Elison *et al.*, 2016	Yes (reviewed by EFSA)	Yes (GRNs 546; 650)

2'-FL = 2'-FL = 2'-O-fucosyllactose GOS = galacto-oligosaccharides; scFOS = short-chain fructo-oligosaccharides

[a] Details of these studies are provided in Section C.

[b] Efficacy endpoints reported in Alliet *et al.* (2016) and Steenhout *et al.* (2016).

[c] Efficacy endpoints reported in Goehring *et al.* (2016).

6. Confidential Commercial Information (CCI)

Confidential commercial information, in relation to food, is defined in Section 4 of the FSANZ Act as meaning:

a) a trade secret relating to food; or

b) any other information relating to food that has a commercial value that would be, or could reasonably be expected to be, destroyed or diminished if the information were disclosed.

Glycom requests the information contained within the following Appendices be considered confidential commercial information.

- Appendix V-a Comprehensive Descriptions of the Manufacturing Processes of 2'-FL and LNnT
- Appendix V-b Documentation on the Development and Safety of the Production Organisms *Escherichia coli* K-12 (DH1) SCR6 and MP572
- Appendix VI-a Internal Methods for Analyses

- Appendix VI-b Internal Methods of Analysis for Detecting 2'-FL and LNnT in Food Matrices
- Appendix VIII Full Study Reports of Unpublished Toxicological Studies
- Appendix IX Full Reports of the Clinical Study Published as Puccio *et al.* (2017)
- Appendix X Bioinformatic Searches for Homology with Known Toxins and Allergens

The information contained within these appendices is not publicly available and release of these data would be at a commercial disadvantage to Glycom. The company has invested considerable capital to develop the production organisms, which efficiently produces high titres of 2'-FL and LNnT that can be isolated as a highly pure material. A non-confidential description of the production organisms and the manufacturing process is provided in Section B.4. Glycom has also invested in the development and validation of the methods of analyses, and commissioned the toxicological studies and infant study that substantiates the safety of their specific ingredients. A non-confidential summary of the unpublished toxicological studies is presented in Sections C.3 and C.4 of this application, while a non-confidential summary of the Puccio *et al.* (2017) infant study is presented in Section C.5.

7. Additional Confidential Information

Glycom requests that the information contained within the following appendices remain confidential:

- Appendix V-c Certificate of Strain Deposition
- Appendix VII Certificates of Analysis

The information contained within these appendices is not publicly available. No other confidential information is included in this application.

8. Exclusive Capturable Commercial Benefit (ECCB)

As indicated in Section A, Glycom is seeking exclusive permission for the use of 2'-FL and LNnT as a novel food. Thus, it is anticipated that this application would confer Exclusive Capturable Commercial Benefit (ECCB) in accordance with Section 8 of the FSANZ Act, which states:

> *An exclusive, capturable commercial benefit is conferred upon a person who applies for the development of a food regulatory measure or the variation of food regulatory measure under Section 22 if:*
>
> *(a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard to draft variation of the standard that would be prepared in relation to the application; and*
>
> *(b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant in order to benefit financially from the approval of the application.*

Additional information to support that this application would confer ECCB, based on the factors of consideration outlined in the Guideline 3.1.1 of the FSANZ Application Handbook (FSANZ, 2016), is summarized in Table 8-1 below.

Table 8-1 Applicability of ECCB to Glycom's Novel Food Application for 2'-FL and LNnT

Factors considered when determining if ECCB is applicable, as outlined in Guideline 3.1.1 of the FSANZ Application Handbook	Response
• Why are you making this application? What are you hoping to get out its approval?	The synthetic oligosaccharides (produced by fermentation) 2'-FL and LNnT are the subject matter of this application. While the manufactured oligosaccharides are identical in structure to the natural oligosaccharides occurring at high concentrations in human breast milk, they do not possess a history of human consumption neither in their manufactured forms, nor as isolated oligosaccharides, and are therefore considered novel foods within the scope of definition of novel food for the purposes of Standard 1.5.1. of the Australia New Zealand Food Standard Code. The purpose of the application herein is to amend Schedule 25 (Permitted Novel Foods) of the Code to include 2'-FL and LNnT obtained by microbial fermentation in the Table to Section S25-2. Furthermore, it is recognised that the approval of 2'-FL and LNnT as a novel food may require consideration and possible changes to the following Standards and their associated Schedules as relevant: • Standard 2.9.1: Infant Formula Products • Standard 2.9.3, Division 4: Formulated Supplementary Foods for Young Children • Schedule 3: Identity and Purity Glycom is hoping to obtain legal permission to market their synthetic 2'-FL and LNnT (from microbiological fermentation) in Australia and New Zealand. In this application, Glycom is requesting for a 15-month exclusivity period should their 2'-FL and LNnT ingredients be approved as novel foods. Glycom and their business partners has significant investment into technology development and specific safety studies of their manufactured forms of 2'-FL and LNnT. It is understood that during the 15-month exclusivity period, ECCB would be conferred.
• How will you benefit from the approval of your application?	Once approval is granted, Glycom will supply 2'-FL and LNnT to their business partners who will commercialise these novel foods in the food categories applied for: • Standard 2.9.1: Infant Formula Products • Standard 2.9.3, Division 4: Formulated Supplementary Foods for Young Children
• Who besides you, will benefit from the approval of your application? How and why will they benefit?	Glycom has exclusive supply agreements with their business partners, who will also benefit from the approval of this application. They will commercialise final food products containing the approved novel food ingredients according to the categories for which approval is being sought for: • Standard 2.9.1: Infant Formula Products • Standard 2.9.3, Division 4: Formulated Supplementary Foods for Young Children The benefit of our business partners may possibly be expressed in context of market shares. It can only be assumed that the product innovation that is connected to the inclusion of the novel food ingredients into final products will have positive impact on market shares.

Table 8-1 Applicability of ECCB to Glycom's Novel Food Application for 2'-FL and LNnT

<table>
<tr><th>Factors considered when determining if ECCB is applicable, as outlined in Guideline 3.1.1 of the FSANZ Application Handbook</th><th>Response</th></tr>
<tr><td></td><td>AUSTRALIA - BRAND A
Total Market Size and % Market Share of Brand A
<table><tr><th>Category</th><th>Total Market Size Vol (Kg) Sales ('000)</th><th>% Market Share Brand A</th></tr><tr><td>Starter Infant Formula</td><td>6206</td><td>3.7</td></tr><tr><td>Follow-on Formula</td><td>4863</td><td>3.4</td></tr><tr><td>Toddler Milk (FSFYC)</td><td>11146</td><td>1.6</td></tr></table>Source: AC Nielsen MAT to 09.04.2017

NEW ZEALAND - BRAND B
Total Market Size and % Market Share of Brand B
<table><tr><th>Category</th><th>Total Market Size Vol (Kg) Sales ('000)</th><th>% Market Share Brand B</th></tr><tr><td>Starter Infant Formula</td><td>629</td><td>11</td></tr><tr><td>Follow-on Formula</td><td>782</td><td>8</td></tr><tr><td>Toddler Milk (FSFYC)</td><td>1279</td><td>12</td></tr></table>Source: AC Nielsen MAT to 28.04.2017</td></tr>
<tr><td>• If your application is approved, whose permission will be required before anyone can derive a benefit from that approval?</td><td>Glycom as the applicant and producer/supplier (only producer according to the technology and specifications described in the application) has to provide permission. Permission is provided in form of specific supply agreements before anyone else can benefit financially.</td></tr>
<tr><td>• Who holds the intellectual property in the subject matter of your application?</td><td>There is no trivial connectivity between the subject matter of the application and intellectual property rights in Australia and New Zealand, or in fact anywhere else:</td></tr>
</table>

Table 8-1 Applicability of ECCB to Glycom's Novel Food Application for 2'-FL and LNnT

Factors considered when determining if ECCB is applicable, as outlined in Guideline 3.1.1 of the FSANZ Application Handbook	Response
	• The oligosaccharides themselves are outside of intellectual property protection since they are identical to natural molecules that have been described in the literature decades ago ("*prior art*"). • The specific food applications sought for approval (i.e. Infant Formula Products and Formulated Supplementary Foods for Young Children) are also not protectable by intellectual property since there is no intellectual novelty recognisable when applying human-identical milk oligosaccharides to these ("*non-obviousness*" and missing "*inventive step*"). • However, some more specific applications are patent protected by Glycom, or their business partners, but these are not the subject matter of this novel food application. • Several aspects of the *technologies used for production* of the oligosaccharides are under patent protection by Glycom, however, potential patent infringements in this area are not easy to monitor/test and thus can hardly be enforced globally. Therefore, Glycom has focussed its patent maintenance on Europe, the United States and China. Currently, Glycom does not possess any longer provisional nor final patent protection in Australia or New Zealand; all previous applications have been abandoned.

9. International and Other National Standards

The national and international standards that are relevant to the current application are listed below. Details of the assessment conducted by other authoritative bodies is presented in Section C.7, while the accepted conditions of use in other jurisdictions are summarised in Section D.5.

Table 9-1 Regulatory Status of 2'-FL and LNnT in Other Jurisdictions

Jurisdiction	Ingredient	Regulatory Status[a]
EU	2'-FL (chemically synthesised)	Glycom's 2'-FL is authorised for use as a novel food ingredient in infant formula and various foods under Commission Implementing Decision (EU) 2016/376 of 11 March 2016 authorising the placing on the market of 2'-O-fucosyllactose as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council. In infant formula and follow-on formula, it may be added at up to **1.2 g 2'-FL/L** in combination with 0.6 g LNnT/L at a ratio of 2:1 in the final ready-to-use product.
	2'-FL (fermentation)	The FSAI has issued a positive opinion that Glycom's 2'-FL obtained by fermentation (as described herein) is substantially equivalent to the 2'-FL that is authorised for use under Commission Decision 2016/376, and this has been successfully notified to the European Commission (EU, 2016a; FSAI, 2016a).
	2'-FL (fermentation)	2'-FL produced by Jennewein Biotechnologie GmbH (Jennewein) is authorised for use as a novel food ingredient in infant formula and follow-on formula at levels of up to **1.2 g 2'-FL/L** (ready-to-use or reconstituted product), under Commission Implementing Decision (EU) 2017/2201 of 27 November 2017 authorising the placing on the market of 2'-fucosyllactose produced with *Escherichia coli* strain BL21 as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council.
	LNnT (chemically synthesised)	Authorised for use as a novel food ingredient in infant formula and various foods under Commission Implementing Decision (EU) 2016/375 of 11 March 2016 authorising the placing on the market of lacto-N-neotetraose as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council. In infant formula and follow-on formula, it may be added at up to **0.6 g LNnT/L** in combination with 1.2 g 2'-FL/L at a ratio of 1:2 in the final ready-to-use product.
	LNnT (fermentation)	FSAI has issued a positive opinion that Glycom's LNnT obtained by fermentation (as described herein) is substantially equivalent to the LNnT that is authorised for use under Commission Decision 2016/375, and this has been successfully notified to the European Commission (EU, 2016b; FSAI, 2016b).
U.S.	2'-FL (chemically synthesised)	FDA has issued a "*no questions*" response on the conclusion that Glycom's 2'-FL ingredient manufactured by chemical synthesis is GRAS for its intended uses in infant formula (up to **2.4 g/L**) and various foods (GRN 546) (U.S. FDA, 2015a).
	2'-FL (fermentation)	FDA has issued a "*no questions*" response on the conclusion that Glycom's 2'-FL ingredient manufactured by fermentation (as described herein) is GRAS for its intended uses in infant formula (up to **2.4 g/L**) and various foods (GRN 650) (U.S. FDA, 2016a)
	2-FL (fermentation)	FDA has issued a "*no questions*" response on the conclusion that 2'-FL obtained by fermentation with *E. coli* BL21 (DE3) strain produced by Jennewein is GRAS for its intended uses in infant formula (up to **2.0 g/L**) (GRN 571) (U.S. FDA, 2015b)
	LNnT (chemically synthesised)	FDA has issued a "no questions" response on the conclusion that Glycom's chemically synthesised LNnT is GRAS for its intended uses in infant formula (**up to 0.6 g/L**) and various foods (GRN 547) (U.S. FDA, 2015c).
	LNnT (fermentation)	FDA has issued a "no questions" response on the conclusion that Glycom's LNnT produced by fermentation (as described herein) is GRAS for its intended uses in infant formula (up to **0.6 g/L**) and various foods (GRN 659) (U.S. FDA, 2016b).
Singapore	2-FL (fermentation)	AVA concluded that Glycom's 2'-FL obtained by fermentation is permitted as an ingredient in infant formula (including follow-on formula) for infants 0 to 12 months old and growing up milk (for children aged 12 to 36 months), up to a level of **1.2 g/L.**
	LNnT (fermentation)	AVA concluded that Glycom's LNnT obtained by fermentation is permitted as an ingredient in infant formula (including follow-on formula) for infants 0 to 12 months old and growing up milk (for children aged 12 to 36 months), up to a level of **0.6 g/L.**
Israel	2-FL (fermentation)	2'-FL obtained by fermentation, as produced by Jennewein Biotechnologie GmbH, has been authorised as a novel food, specifically for use in milk-based infant formulas (ages 0 to 6 months) and follow-on formulas (ages 6 to 12 months), at levels of up to **2.0 g/L**.

EU = European Union; FDA = Food and Drug Administration; FSAI = Food Safety Authority of Ireland; GRAS = Generally Recognized as Safe; U.S. = United States.
[a] The use levels of 2'-FL and LNnT in infant formula and follow-on formula that are cited in this table refer to the amount present in the reconstituted or ready-to-feed formula (*i.e.*, as consumed).

10. Statutory Declaration

A signed Statutory Declaration is provided in Appendix I.

11. Checklist

A completed checklist relating to the information required for submission with this application is provided in Appendix II.

A. EXCLUSIVE USE OF NOVEL FOODS

This section is completed in accordance with Section A of Guideline 3.5.2 – Novel Foods of the Food Standards Australia New Zealand Application Handbook (FSANZ, 2016), which states the following information are to be provided:

1. A statement as to whether the application is seeking exclusive permission for the novel food. If exclusive permission is sought, the application must include details of the following:

    a. the specific class of food; and

    b. the brand of the food, including the name the food will be marketed under (if known).

Glycom is seeking exclusive permission for the use of 2'-FL (either alone or in combination with LNnT) as a novel food on the basis that the 2'-FL and LNnT are highly refined products obtained *via* proprietary manufacturing processes. There have been significant research and investment by Glycom and its partners into the development of 2'-FL and LNnT. It is envisioned that exclusivity will be specific to Glycom's 2'-FL and LNnT when they are used as food ingredients, and not to finished food products containing these materials. In practice, this means that during the exclusivity period, a manufacturer may incorporate 2'-FL (either alone or in combination with LNnT) into their food products only if they obtain the ingredient from Glycom, and provided that the use is in accordance with the agreed conditions specified in the approval from FSANZ. Specifically, Glycom is seeking exclusivity for the following:

Class of Food: Human-identical milk oligosaccharide (HiMO)

Brand of the Food: not available yet[3]

[3] The brand name under that 2'-FL (either alone or in combination with LNnT) will be marketed in Australia/New Zealand will be provided to FSANZ once this is known.

B. TECHNICAL INFORMATION ON THE NOVEL FOOD

Technical information on Glycom's 2'-FL and LNnT ingredients are described in this Section. Specifically, this section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel Foods) and Guideline 3.3.2 (Processing Aids) of the Food Standards Australia New Zealand Application Handbook (FSANZ, 2016). The corresponding Sections of this Application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	**Required Information Described in the Guideline**	**Section of the Application where this is Addressed**
Guideline 3.5.2 – Novel Foods	***B. Technical information on the novel food***	
	B.1 Information on the type of novel food	Section B.1
	B.2 Information on the purpose of adding a novel food ingredient to food	Section B.2
	B.3 Information on the physical and chemical properties of the novel food or novel food ingredient	Section B.3
	B.4 Information on the impurity profile for a typical preparation	Section B.5
	B.5 Manufacturing process for a novel food ingredient	Section B.4
	B.6 Specification for identity and purity for a novel food ingredient	Section B.6
	B.7 Analytical method for detection of a novel food ingredient	Section B.8
Guideline 3.3.2 – Processing Aids	***D. Additional information related to the safety of an enzyme processing aid derived from a microorganism***	
	D.1 Information on the source microorganism	Section B.4.3
	D.2 Information on the pathogenicity and toxicity of the source microorganism	Section B.4.3.3
	D.3 Information on the genetic stability of the source organism	Section B.4.3.4
	E. Additional information related to the safety of an enzyme processing aid derived from a genetically-modified microorganism	
	E.1 Information on the methods used in the genetic modification of the source organism	Section B.4.3.2

B.1 Information on the Type of Novel Food

Glycom obtains 2'-FL from the fermentation with an *Escherichia coli* K-12-derived strain that has been optimised for the biosynthesis of 2'-FL. The 2'-FL is secreted into the fermentation medium. The *E. coli* cells are removed by filtration, and as described further in Section B.5, a number of further purification steps are applied to result in a single, isolated, high-purity crystalline ingredient that is specified to contain a minimum of 94% of 2'-FL (and a minimum of 96% of the sum of human-identical milk saccharides). 2'-FL occurs only as one specific constitutional isomer.

To produce LNnT, a separate, independent fermentation process has also been developed by Glycom. This production process is similar to the one used to manufacture 2'-FL. LNnT is secreted into the fermentation medium by the *E. coli* K-12-derived strain that has been optimised for the biosynthesis of LNnT. The *E. coli* cells are removed by filtration, and as described further in Section B.5, a number of further purification steps are applied to result in a single, isolated, high-purity crystalline ingredient that is specified to contain a minimum of 92.0% of LNnT (and a minimum of 95% of the sum of human-identical milk saccharides). LNnT also occurs only as one specific constitutional isomer.

Therefore, of the major novel food categories listed in Section 3.5.2 – Novel Foods of the Food Standards Australia New Zealand *Application Handbook* (FSANZ, 2016), Glycom's 2'-FL and LNnT ingredients are most appropriately classified as:

(IV) Single chemical entities.

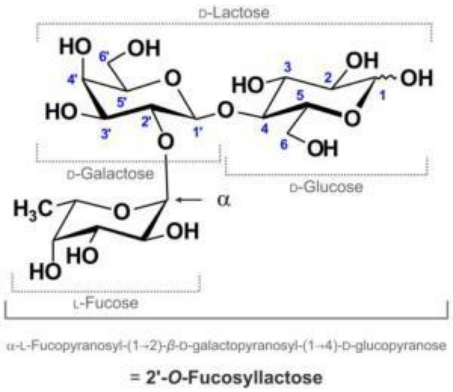
B.2 Information on the Purpose of Adding a Novel Food Ingredient to Food

2'-FL (either alone or in combination with LNnT) is intended for addition to infant formula products and formulated supplementary foods for young children. A detailed discussion of the purpose for adding 2'-FL (either alone or in combination with LNnT) to these products is presented in Section E.

In brief, the addition of 2'-FL and LNnT to infant formula and follow-on formula is intended to result in products that better reflect the compositional profile of oligosaccharides of human breast milk. This is consistent with efforts to produce formula products that better match the nutrient composition of human milk, as set forth by principles in the Australia and New Zealand Food Regulation Ministerial Council's *Policy Guideline on the Regulation of Infant Formula Products* and the Codex *Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants* (Codex Stan 72). Given their unique presence and abundance in human breast milk, the physiological roles of 2'-FL and LNnT have been investigated in a number of preclinical and clinical studies (see Section E). HMOs including 2'-FL and LNnT are non-digestible oligosaccharides; their ability to positively modulate the commensal microflora within the gastrointestinal tract (*i.e.*, exert bifidogenic properties) has been well established, which has important implications on the hosts' health. Other mechanisms have also been proposed by which HMOs may be beneficial, including their ability to adhere to pathogens, and improve intestinal barrier function and immune modulation (see Section E). Therefore, the proposed uses of 2'-FL and LNnT in Australia/New Zealand could serve to benefit both infants and young children alike.

B.3 Information on the Physical and Chemical Properties of the Novel Food Ingredient

B.3.1 Identity of 2'-FL

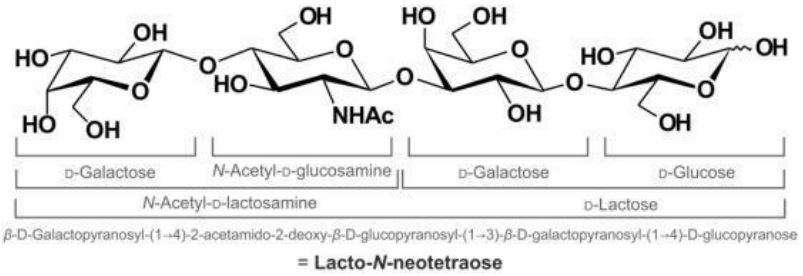
Common Name:	2'-*O*-Fucosyllactose
Common Abbreviations:	2'-FL (2'FL, 2-FL, 2FL)
International Union of Pure and Applied Chemistry (IUPAC) Name:	α-D-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Alternative Denotations:	2'-*O*-L-Fucosyl-D-lactose; Fucosyl-α-1,2-galactosyl-β-1,4-glucose; Fuc-α-(1→2)-Gal-β-(1→4)-Glc
Chemical Abstracts Service (CAS) Registry Number:	41263-94-9
Chemical Formula:	$C_{18}H_{32}O_{15}$
Molecular Weight:	488.44
Structural Formula:	

2'-FL is a naturally occurring trisaccharide consisting of L-fucose, D-galactose, and D-glucose. Alternatively, the molecular constitution can be described as consisting of the monosaccharide L-fucose and the disaccharide D-lactose, which are linked by an alpha (1→2) bond to form the trisaccharide. 2'-FL occurs only as one specific constitutional isomer.

The molecular structure of 2'-FL was elucidated for the first time using classical chemical techniques by Richard Kuhn in 1955 (Kuhn *et al.*, 1955), and shortly after by Jean Montreuil (Montreuil, 1956). Since then, the structure has been independently confirmed by a range of modern structure characterisation techniques, including spectroscopic techniques [*e.g.*, ^{1}H-, ^{13}C, and 2D-nuclear magnetic resonance (NMR)] (Jenkins *et al.*, 1984; Ishizuka *et al.*, 1999; Rundlöf *et al.*, 2001; Urashima *et al.*, 2002, 2004, 2005; Almond *et al.*, 2004; Wada *et al.*, 2008), mass spectrometric (MS) techniques (Fura and Leary, 1993; Asres and Perreault, 1996; Perreault and Costello, 1999), and X-ray crystallography (Kuhn *et al*., 1956; Svensson *et al*., 2002).

2'-FL produced by fermentation with an *E. coli* K-12-derived strain is chemically and structurally identical to the 2'-FL present in human breast milk, as confirmed by ^{1}H- and 2D-NMR-spectroscopy, mass spectrometry and x-ray crystallography (see Appendix IV for analytical report).

B.3.2 Identity of LNnT

Common Name:	Lacto-*N*-neotetraose
Common Abbreviation:	LNnT
IUPAC Name:	β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose
Alternative Denotations:	Gal-β-(1→4)-GlcNAc-β-(1→3)-Gal-β-(1→4)-Glc *N*-Acetyl-D-lactosamine-β-(1→3)-D-lactose
Chemical Abstracts Service (CAS) Registry Number:	13007-32-4
Chemical Formula:	$C_{26}H_{45}NO_{21}$
Molecular Weight:	707.63
Structural Formula:	 β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose = **Lacto-*N*-neotetraose**

LNnT is a naturally occurring tetrasaccharide consisting of D-galactose, *N*-acetyl-D-glucosamine, D-galactose and D-glucose. It occurs only as one specific constitutional isomer. The molecular structure of LNnT was characterised by Richard Kuhn in 1962, and since then a number of publications reported detailed structure characterisation by ^{1}H- and ^{13}C-NMR techniques (Strecker *et al.*, 1989; Urashima *et al.*, 2002, 2005).

LNnT produced by fermentation with an *E. coli* K-12-derived strain is chemically and structurally identical to LNnT that is present in human breast milk, as confirmed by ^{1}H- and 2D-NMR-spectroscopy, mass spectrometry and x-ray crystallography (see Appendix IV for analytical report).

B.4 Manufacturing Process

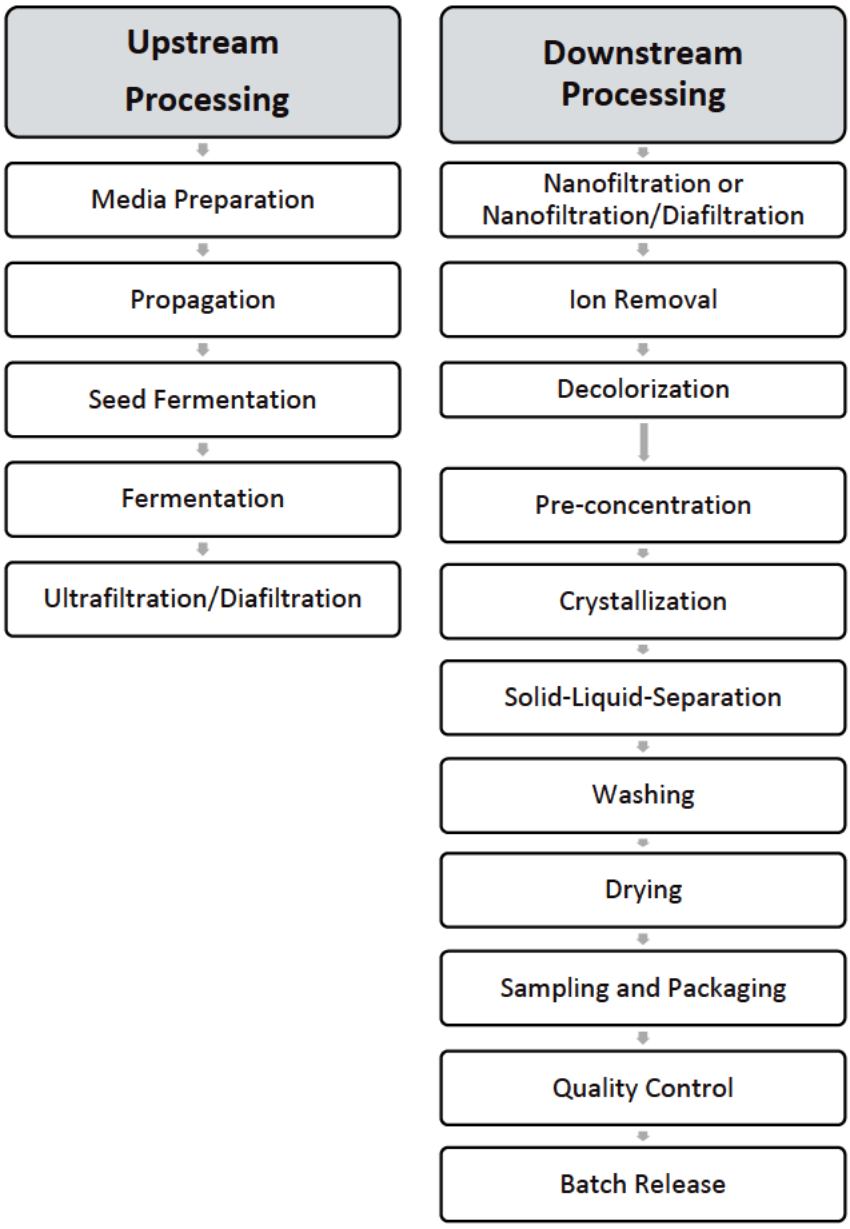
B.4.1 Manufacturing Process for 2'-FL

B.4.1.1 Overview

Glycom's 2'-FL ingredient is currently manufactured in compliance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The manufacturing process can be broadly divided into 2 stages. In Stage 1 [upstream processing (USP)], D-lactose and D-sucrose are converted to 2'-FL by the cellular enzymes of the 2'-FL production microorganism, which uses D-sucrose as an exclusive energy and carbon source and D-lactose as a substrate for 2'-FL biosynthesis. In Stage 2 [downstream processing (DSP)], a series of purification and isolation steps are used to generate the final high-purity 2'-FL ingredient. A schematic overview of the manufacturing process for 2'-FL is presented in Figure B.4.1.1-1 below. Confidential details on the production process are provided in Appendix V-a.

To note, Glycom's 2'-FL ingredient is currently manufactured in Denmark/the European Union, it will not itself be manufactured in Australia or New Zealand, thus the fermentation substrates, organism and processing aids used for its manufacture will not enter the territory.

Figure B.4.1.1-1 Overview of the Manufacturing Process for 2'-FL



B.4.1.2 Identity of Raw Materials and Processing Aids

The raw materials/processing-aids used to make 2'-FL are D-lactose and D-sucrose (both meeting the specifications established in the European Pharmacopoeia). These reagents are sterilised before use and are subject to quality control pre-screening using High-Performance Liquid Chromatography (HPLC) for purity (*e.g.*, lactose can isomerise to lactulose through the Lobry de Bruyn–van Ekenstein aldose-ketose isomerisation). Fermentation is performed in a chemically-defined, salt-based, minimal growth medium with D-sucrose as the only carbon source and D-lactose as the substrate. All processing aids, raw materials, unit operations and filter aids have been sourced with Halal certificates, and the production line and ingredient will be certified as Halal by the Halal Food Council of Europe.

The materials used in the production of 2'-FL by fermentation are listed in Table B.4.1.2-1.

Table B.4.1.2-1 Raw Materials and Processing Aids Used in the Manufacture of 2'-FL

Material	Function
Raw Material Substrates (Bioreagents)	
D-Sucrose	Carbon and energy source
D-Lactose	Substrate/source raw material
Other Components of Fermentation Medium	
Ammonium hydroxide	Fermentation medium ingredient (nitrogen source)
Magnesium sulphate	Fermentation medium ingredient (essential element)
Ammonium dihydrogen phosphate	Fermentation medium ingredient (nitrogen and phosphate source)
Potassium dihydrogen phosphate	Fermentation medium ingredient (essential element and phosphate)
Potassium hydroxide	pH adjustment
Sodium hydroxide	pH adjustment
Citric acid	Fermentation medium ingredient (essential element)
Thiamine	Fermentation medium ingredient (vitamin growth factor)
Minerals	Fermentation medium ingredient (essential elements)
Processing Aids	
Acetic acid	Crystallisation
Food-grade anti-foaming agent	Anti-foaming agent
Filters and Filtration Aids	
Ultrafiltration	Removal of cell matter and proteins
Nanofiltration	Removal of small molecules
Ion-exchange resin	Removal of small molecules
Electrodialysis	Removal of small charged molecules (*e.g.*, salts)
Charcoal filter	Decolourisation and removal of impurities
Microfiltration	Filter sterilisation

2'-FL = 2'-*O*-Fucosyllactose

B.4.1.3 Details of the Manufacturing Steps

Manufacturing Stage 1: Fermentation Procedure

Fermentation is performed in a chemically-defined, salt-based, minimal medium, with D-sucrose as the only carbon source and D-lactose as the substrate. Fermentation is maintained for several days until in-process quality controls indicate a favourable ratio of 2'-FL to other carbohydrates, as well as high consumption of D-lactose.

2'-FL is efficiently excreted into the fermentation broth; therefore, disruption of the cells is not required for isolation of 2'-FL from the culture broth. The microbial biomass containing the intact production organism is then removed from the culture supernatant containing 2'-FL by an ultrafiltration/ diafiltration aid and the separated microbial biomass is deactivated by heat treatment. The quality of the clear ultrafiltration/diafiltration permeate is assessed by a range of in-process quality controls and then further purified in the second stage of the production process, the downstream processing.

Manufacturing Stage 2: Purification and Isolation

Stage 2 of the manufacturing process consists of a series of purification steps, most notably the final selective crystallisation step, that generates the single, isolated, high-purity, crystalline trisaccharide 2'-FL ingredient. During this stage, water, minerals, small molecules, and other potential impurities are removed by nanofiltration, ion-exchange resin, and activated charcoal filtration, followed by concentration by nanofiltration and highly controlled crystallisation with the use of defined amounts of acetic acid. These resulting crystals are dried until the required specification limit for acetic acid is met.

Quality control measures are in place during the entire purification and isolation process to ensure that final batches of 2'-FL released conform to the product specifications.

B.4.1.4 Quality Control

As previously mentioned, the manufacture of 2'-FL is conducted in accordance with GMP and HACCP principles. Both manufacturing stages are controlled by a HACCP plan which includes specifications for the equipment, raw materials, product, and packaging materials used in the manufacturing process. The manufacture of 2'-FL employs a number of in-process controls to ensure the purity of the final product and minimise the amount of potential inherent impurities to the level that is technically feasible.

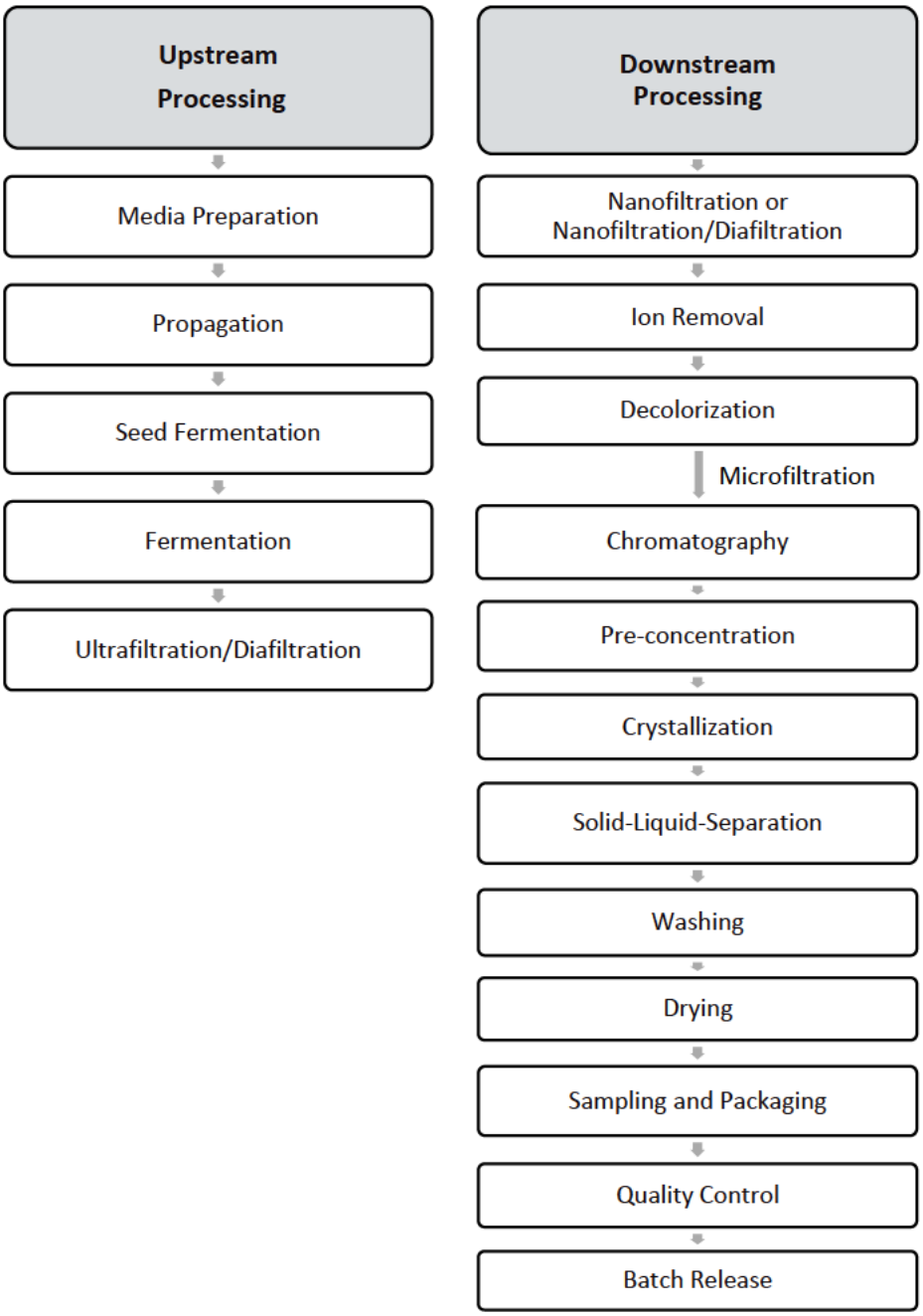
B.4.2 Manufacturing Process for LNnT

B.4.2.1 Overview

LNnT is manufactured in compliance with cGMP and HACCP principles. The raw materials from which LNnT is derived include D-lactose as a substrate, and D-glucose and D-glycerol as energy- and carbon-sources in a fermentation process. The manufacturing process can be broadly divided into 2 stages: in Stage 1 USP), D-lactose is converted *via* the metabolic intermediate "lacto-*N*-triose II" to LNnT by the cellular enzymes of the LNnT production organism. In Stage 2, (DSP), a series of purification and isolation steps generate the final high-purity LNnT product. A schematic overview of the manufacturing process for LNnT is presented in Figure B.4.2.1-1 below. Confidential details of the production process are provided in Appendix V-a.

To note, Glycom's LNnT ingredient is currently manufactured in Denmark/the European Union, it will not itself be manufactured in Australia or New Zealand, thus the fermentation substrates, organism and processing aids used for its manufacture will not enter the territory.

Figure B.4.2.1-1 Overview of the Manufacturing Process for LNnT



B.4.2.2 Identity of Raw Materials and Processing Aids

The D-lactose, D-glucose and D-glycerol raw materials used meet the specifications established in the European Pharmacopeia or food grade equivalent standard. The raw materials are sterilised before use and monitored by HPLC for potential side products (*e.g.*, lactose can isomerise to lactulose through the well-known aldose-ketose isomerisation). Fermentation is performed in a chemically well-defined, salt-based, minimal medium. D-Glucose, D-glycerol and ammonium salts are used as carbon and nitrogen sources and D-lactose serves as a substrate for LNnT synthesis. All processing aids, raw materials, unit operations and filter aids have been sourced with Halal certificates, and the production line and ingredient will be certified as Halal by the Halal Food Council of Europe.

The materials used in the production of LNnT are listed in Table B.4.2.2-1.

Table B.4.2.2-1 Raw Materials and Processing Aids Used in the Manufacture of LNnT

Material	Function
Raw Material Substrates (Bioreagents)	
D-lactose	Substrate/source raw material
D-glucose	Energy/carbon source
D-glycerol	Energy/carbon source
Other Components of Fermentation Medium	
Ammonium hydroxide	Fermentation medium ingredient (nitrogen source)
Magnesium sulphate	Fermentation medium ingredient (essential element)
Ammonium dihydrogen phosphate	Fermentation medium ingredient (nitrogen and phosphate source)
Potassium dihydrogen phosphate	Fermentation medium ingredient (essential element and phosphate)
Potassium hydroxide	pH adjustment
Sodium hydroxide	pH adjustment
Citric acid	Fermentation medium ingredient (essential element)
Thiamine	Fermentation medium ingredient (vitamin growth factor)
Minerals	Fermentation medium ingredient (essential elements)
Processing Aids	
Methanol	Crystallisation
Food-grade anti-foaming agent	Anti-foaming agent
Filters and Filtration Aids	
Ultrafiltration	Removal of cell matter and proteins
Nanofiltration	Removal of small molecules
Ion-exchange resin	Removal of small molecules
Electrodialysis	Removal of small charged molecules (*e.g.*, salts)
Charcoal filter	Decolourisation and removal of impurities
Microfiltration	Filter sterilisation

LNnT = lacto-*N*-neotetraose.

B.4.1.3 Details of the Manufacturing Steps

Manufacturing Stage 1: Fermentation Procedure

During Stage 1, D-lactose (substrate), D-glucose, and D-glycerol (carbon and energy source) are converted into LNnT by the production organism. The fermentation is maintained for several days until in-process controls indicate a favourable ratio LNnT to other carbohydrates, and a high consumption of D-lactose.

LNnT is excreted into the fermentation broth; therefore, disruption of the cells is not required for isolation of LNnT from the culture broth. The microbial biomass containing the production organism is then removed from the culture supernatant containing LNnT by ultrafiltration/ diafiltration and the separated microbial biomass is deactivated by heat treatment. The quality of the clear ultrafiltration/diafiltration permeate is assessed by a range of in-process controls and then further purified by the second stage of the production process, the downstream processing.

Manufacturing Stage 2: Purification and Isolation

Stage 2 of the manufacturing process consists of a series of purification steps, most notably the final selective crystallisation step, that generate the single, isolated, high-purity, crystalline tetrasaccharide LNnT ingredient. During this stage, water, minerals, small molecules, and other potential impurities are removed by nanofiltration, ion-exchange resin/electrodialysis, activated charcoal filtration, and chromatography, followed by concentration by vacuum distillation or nanofiltration and crystallisation with the use of highly controlled amounts of methanol. The crystals are then dried. Quality control measures are in place during the entire purification and isolation process to ensure that final batches of LNnT released conform to the product specifications.

B.4.2.4 Quality Control

As previously indicated, the manufacture of LNnT is conducted in accordance with GMP and HACCP principles. Both manufacturing stages are controlled by a HACCP plan which includes specifications for equipment, raw materials, product, and packaging materials. The manufacture of LNnT employs a number of in-process controls to ensure the purity of the final product and minimise the amount of potential inherent impurities to the level that is technically feasible.

B.4.3 Description of the Source Microorganism

B.4.3.1 Origins and History of Use

Comprehensive documentation on the production organism, including its development, historical use, and information on its safety as a production strain is provided in Appendix V-b. Briefly, the fermentation organisms used in the production of both 2'-FL and LNnT are derived from *E. coli* K-12. *E. coli* K-12 has been specifically developed and recognised as a "safety strain" for molecular biological research in the 1970s (Manning *et al.*, 1977; Smith, 1978) and is since then the most widest applied microorganism in biotechnology research laboratories around the world. In 1997, it was also among the first organisms in history of modern sequencing technologies for which the whole genome sequence became available (Blattner *et al.*, 1997). Recent comparison of sequenced *E. coli* genomes shows that K-12 and closely related "safety strains" of *E. coli* possess 10 to 20% less genes than their pathogenic cousins (Lukjancenko *et al.*, 2010). *E. coli* K 12-derived strains cannot colonise in the human gastrointestinal system, possess a strongly reduced ability for survival in the environment, and do not produce protein-type toxins (U.S. EPA, 1997). *E. coli* K-12 is today one of the preferred microorganisms for industrial biotechnology with wide application scope (Chen *et al.*, 2013; Theisen and Liao, 2017).

The safety of *E. coli* derivatives for use in industrial applications has been comprehensively reviewed in the United States Environmental Protection Agency (U.S. EPA) *Final Risk Assessment of Escherichia coli K-12 Derivatives* (U.S. EPA, 1997). The host strain *E. coli* K-12 and its derivatives are generally recognised as safe and suitable for use as a host organism in the construction of biotechnologically engineered microorganisms used for the industrial production of food ingredients, food enzymes, food additives and biochemical (such as biofuels, organic acids, amino acids, sugar alcohols and biopolymers) (Chen *et al.*, 2013; Theisen and Liao, 2017). For example, in the EU, *E. coli* K-12 has been recommended as a safe host organism by the EU Commission and derivatives have been repeatedly assessed by the European Food Safety Authority (EFSA) as safe for the production of food and feed ingredients, additives, and food

enzymes [*e.g.,* chymosin (JECFA, 2010), gamma cyclodextrin (ACNFP, 2012), L-methionine (EFSA, 2013), L-valine (EFSA, 2008), L-threonine (EFSA, 2014a), L-lysine (EFSA, 2014b), L-isoleucine (EFSA, 2010), L-tryptophan (EFSA, 2016)]. Additionally, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has assessed *E. coli* K-12 as a safe host for food enzyme preparations [*e.g.*, chymosin (JECFA, 1991, 2006)] and the French government explicitly lists chymosin derived from *E. coli* K-12 fermentation as an approved processing aid (see www.legifrance.gouv.fr) (Legifrance, 2006). Moreover, *E. coli* K-12 has been approved for the production of Chymosin A in China by the MOH (Ministry of Health, now National Health and Family Planning Commission) in GB 2760-2014 (Ministry of Health of the PRC, 2014). In the U.S., *E. coli* K-12 and B-strain derivatives are used in the production of a range of GRAS ingredients and food enzymes [*e.g.,* alpha cyclodextrin (U.S. FDA, 2004), chymosin (U.S. FDA, 2016c), L-leucine (U.S. FDA, 2010), and β-galactosidase (U.S. FDA, 2014)].

Other food ingredients and/or food additives produced with *E. coli* K-12-derived strains include xylitol (Khankal *et al.*, 2008; von Rymon Lipinski, 2014), thaumatin (Daniell *et al.*, 2000; von Rymon Lipinski, 2014), tagatose (Roh *et al.*, 2000; von Rymon Lipinski, 2014), formic acid (Murarka *et al.*, 2008; Shams Yazdani and Gonzalez, 2008), L-phenylalanine (Karau and Grayson, 2014), L-tyrosine (Karau and Grayson, 2014), and L-valine (Karau and Grayson, 2014).

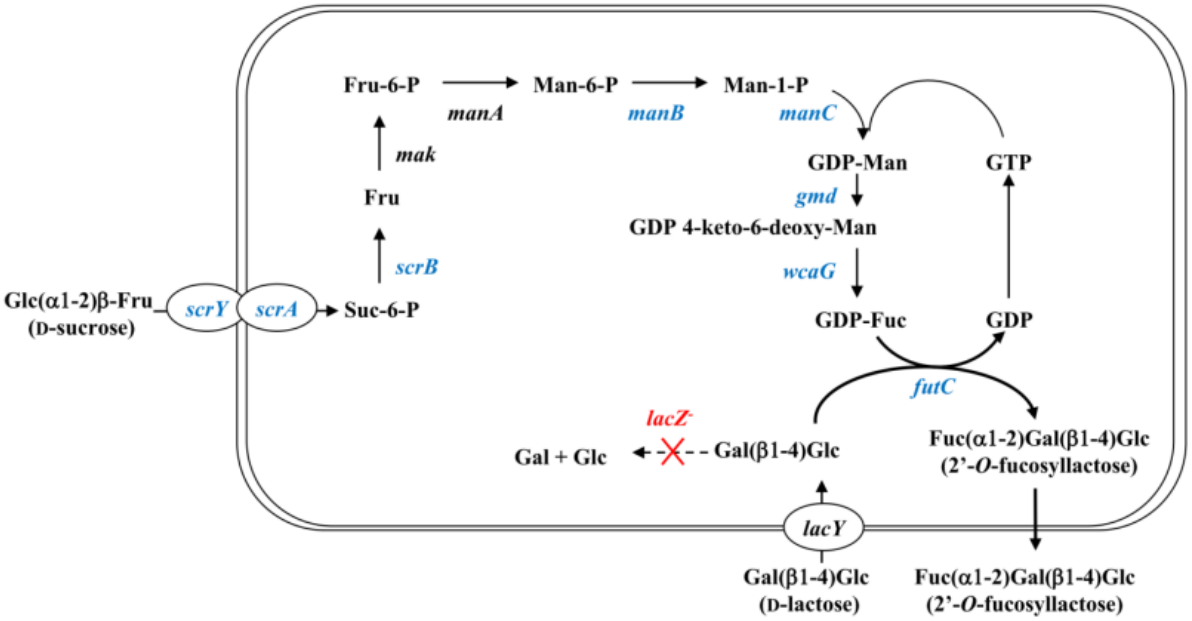
B.4.3.2 Information on the Genetic Modifications of the Source Microorganism

Production of 2'-FL

The fermentation organism for the production of 2'-FL is *E. coli* K-12 (DH1) SCR6, a highly stable and reliable production strain that provides high titres of 2'-FL. A comprehensive description of the genetic modifications and steps taken to construct the final production strain is provided in Appendix V-b. The strain has been deposited in the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) in Braunschweig, Germany (see Appendix V-c).

It is important to recognise that the genetic modifications made to production organism are intended to introduce the metabolic pathway to allow for 2'-FL biosynthesis. The newly expressed proteins are not the material of commercial interest; rather, it is the substance (*i.e.*, 2'-FL) that is synthesised by these newly expressed proteins. Figure B.4.3.2-1 shows the biochemical pathway by which the production strain generates 2'-FL using D-lactose and D-sucrose as a substrate and carbon source, respectively. During manufacture, the production strain remains intact, secretes the 2'-FL, and then is entirely removed through a series of purification steps.

Figure B.4.3.2-1 Pathway for 2'-FL Biosynthesis by *Escherichia coli* K-12 (DH1) SCR6

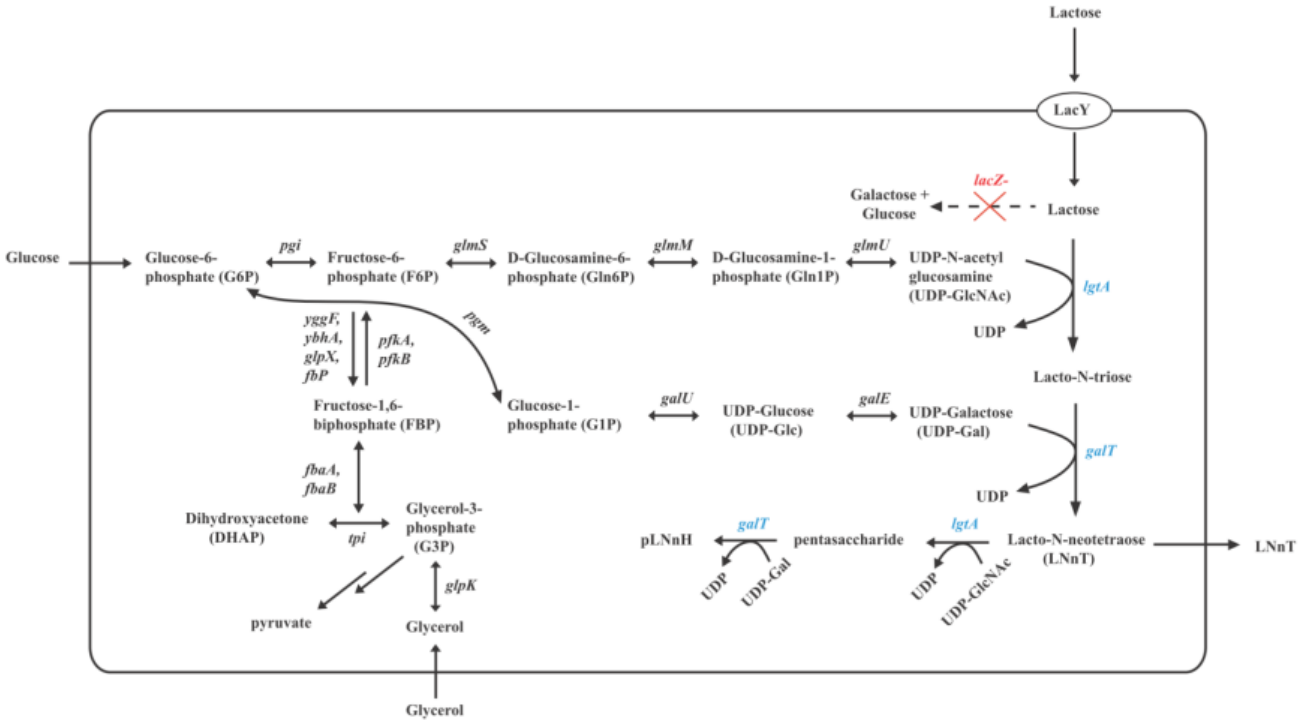


Production of LNnT

The fermentation organism for the production of LNnT is *E. coli* K-12 (DH1) MP572, a highly stable and reliable production strain that provides high titres of LNnT. A comprehensive description of the genetic modifications and steps taken to construct the final production strain is provided in Appendix V-b. The strain has been deposited in the DSMZ in Braunschweig, Germany (see Appendix V-c).

It is important to recognise that the genetic modifications made to production organism are intended to introduce the metabolic pathway to allow for LNnT biosynthesis. The newly expressed proteins are not the material of commercial interest; rather, it is the substance (*i.e.*, LNnT) that is synthesised by these newly expressed proteins. Figure B.4.3.2-2 shows the biochemical pathway by which the production strain generates LNnT using lactose, glucose, and glycerol. During manufacture, the production strain remains intact, secretes the LNnT, and then is entirely removed through a series of purification steps.

Figure B.4.3.2-2 Pathway for LNnT Biosynthesis by *Escherichia coli* K-12 (DH1) MP572



B.4.3.3 Information on the Pathogenicity and Toxicity of the Source Microorganism

E. coli K-12 (and its derivative DH1) is defective in at least three cell wall characteristics, making it unable to colonise the human intestinal tract under normal conditions. Experiments in humans and animals have confirmed that the K-12 strain does not colonise the intestine and moreover that indigenous intestinal microorganisms have a competitive advantage (U.S. EPA, 1997). Furthermore, the K-12 strain does not possess adhesion properties or virulence factors and thus does not meet the requirements for pathogenicity.

With regards to toxigenicity, K-12 strain does not produce significant quantities of toxins that affect humans. No antibiotic production from *E. coli* K-12 nor its derivative DH1 have been characterised to date.

B.4.3.4 Information on the Genetic Stability of the Source Microorganism

The *E. coli* K-12 (DH1) strain used in the production of 2'-FL and LNnT carries the *recA1* genotype, which has a significantly reduced homologous recombination rate of the plasmid DNA. Secondly, the plasmids used in the production strain carry metabolic markers (*i.e.*, sucrose operon and the *nadC* gene; for details see Appendix V-b), so that by careful design of the growth media used during cell banking, propagation and fermentation, the production strains are protected from plasmid loss.

Full details of the measures taken by Glycom to ensure genetic stability of the production strain is provided in Appendix V-b. To help ensure that the cell line is preserved, Glycom uses a two-tiered cell banking system where a master cell bank (MCB) is used to generate the working cell banks (WCB) that are in turn used to start the production batches. Such a cell banking system is generally accepted as the most practical approach to providing sufficient quantities of cells for continued manufacture of the product, and warrants that each and every fermentation is performed from a consistent basis. The MCB and WCB must meet a number of acceptance criteria to ensure purity, productivity and quality prior to

their use. The MCB is derived from an initial cell clone (ICC), which is generated from a mother clone vial; the mother clone is deposited externally and is also stored internally in a secured -80°C freezer. Glycom conducts fermentation performance tests to ensure that the ICC is stable for at least the number of generations that is expected to be needed for production use (see Appendix V-b). A comprehensive dataset is available to demonstrate that the source microorganism does not undergo strain drift, and that the culture conditions can be consistently applied across batches (see Appendix V-b).

Additionally, a number of quality control steps are employed during the production process of 2'-FL and LNnT. The following are used as indication of the genetic stability of the production strain:

1) Cell growth parameters have to be within historical range;
2) Titres have to be within historical range;
3) The final 2'-FL and LNnT product exhibits a consistent impurity profile and is compliant with the specifications defined in Section B.6.1.

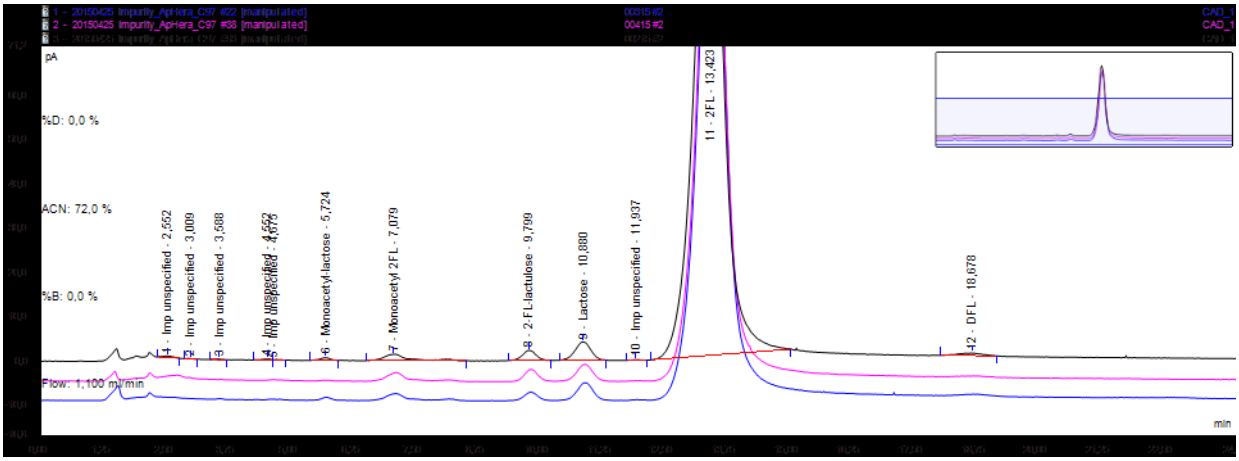
B.5 Information on the Impurity Profile for a Typical Preparation

B.5.1 Carbohydrate Impurity Profile

B.5.1.1 Carbohydrate Impurity Profile for 2'-FL

Batch analyses from multiple lots of 2'-FL have been analysed using HPLC, High-Performance Anion Exchange Chromatography (HPAEC) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS). All batches displayed as similar impurity profile demonstrating consistency of the manufacturing process. Figure B.5.1.1-1 shows a representative impurity profile of several batches as measured by HPLC using a Corona Charged Aerosol Detector (cCAD). The main advantages of the detector used are high sensitivity, wide dynamic detection range, consistent performance with excellent precision and more consistent response over wide range of chemical structures (*e.g.*, no chromophores are required for detection).

Figure B.5.1.1-1 Representative Impurity Profile of 2'-FL as Measured by High Performance Liquid Chromatography and Corona Charged Aerosol Detector



Results of the HPLC-cCAD analyses demonstrate that saccharides that are structurally related to 2'-FL can be present at small quantities in the final isolated product, namely D-lactose (not more than 3%), L-fucose (not more than 1%), difucoslylactose (not more than 1%) and 2'-fucosyl-D-lactulose (not more than 1.0%). The first 3 (lactose, fucose and difucosyllactose) are all natural components of breastmilk

and the resulting exposure from the levels in 2'-FL would be insignificant compared to the exposure from each of these saccharides at the naturally occurring levels.

2'-Fucosyl-D-lactulose is an isomerisation product of 2'-FL where the terminal glucose moiety is converted into a fructose sugar. This type of isomerisation is pH and temperature dependent and has been commonly reported for the closely related conversion of D-lactose into D-lactulose during heat treatment [*i.e.*, ultra-high temperature (UHT) processing and pasteurisation] of milk, including human donor milk (Beach and Menzies, 1983; Schuster-Wolff-Bühring *et al.*, 2010; Gómez de Segura *et al.*, 2012). This isomerisation reaction of carbohydrates is also known as the Lobry de Bruyn–van Ekenstein transformation (Angyal, 2001; Wang, 2010). Different infant formulas have been reported to contain D-lactulose at relative levels between 1 and 7% of their D-lactose content, and absolute levels up to 13.7 mmol/L (Beach and Menzies, 1983). Although the isomerisation product of 2'-FL has not been specifically evaluated in heat treated human donor milk, D-lactulose has also been detected at significant proportions of D-lactose (Gómez de Segura *et al.*, 2012), and it can thus be reasonably assumed that 2'-fucosyl-D-lactulose is present at comparable ratios and can thereby be equally regarded to have a history of safe use from heat treated human donor milk.

B.5.1.2 Carbohydrate Impurity Profile for LNnT

Batch analyses from multiple lots of LNnT have been analysed using HPLC-cCAD and all batches exhibit comparable impurity profiles, thereby demonstrating consistency of the manufacturing process. Figure B.5.1.2-1 shows a representative impurity profile for LNnT and Figure B.5.1.2-2 presents the results of 3 batches analysed for impurities. Small amounts of carbohydrate-type impurities may be detected in the LNnT ingredient; however, these compounds are HiMOs themselves or fall into the general structural patterns observed in HMOs. Such carbohydrate-type compounds that may form during the fermentation process include lacto-*N*-triose II, *para*-lacto-*N*-neohexaose, and LNnT fructose isomer. The LNnT fructose isomer is an isomerisation product of LNnT where the terminal glucose moiety is converted into a fructose sugar; this isomerisation is also described as the Lobry de Bruyn–van Ekenstein transformation as mentioned previously. As with 2'-FL, it can be reasonably assumed that the LNnT fructose isomer is present at comparable ratios to that of heat-treated human milk and can therefore be equally regarded to have a history of safe consumption.

Figure B.5.1.2-1 Representative Impurity Profile for LNnT, as Measured by High Performance Liquid Chromatography with a Corona Charged Aerosol Detector

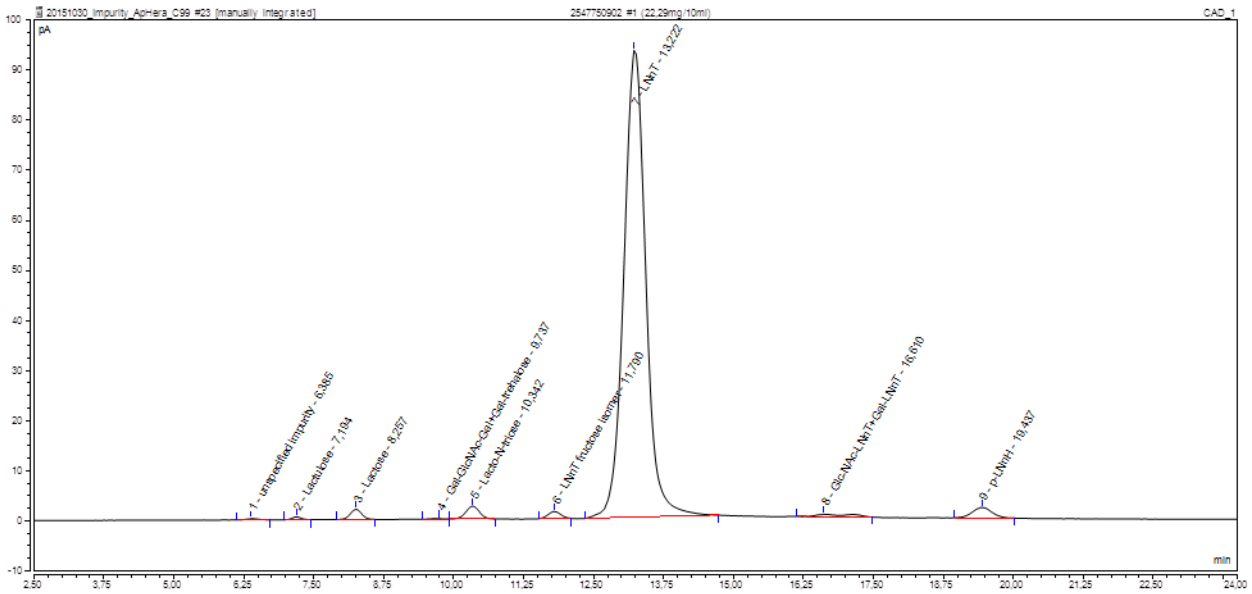
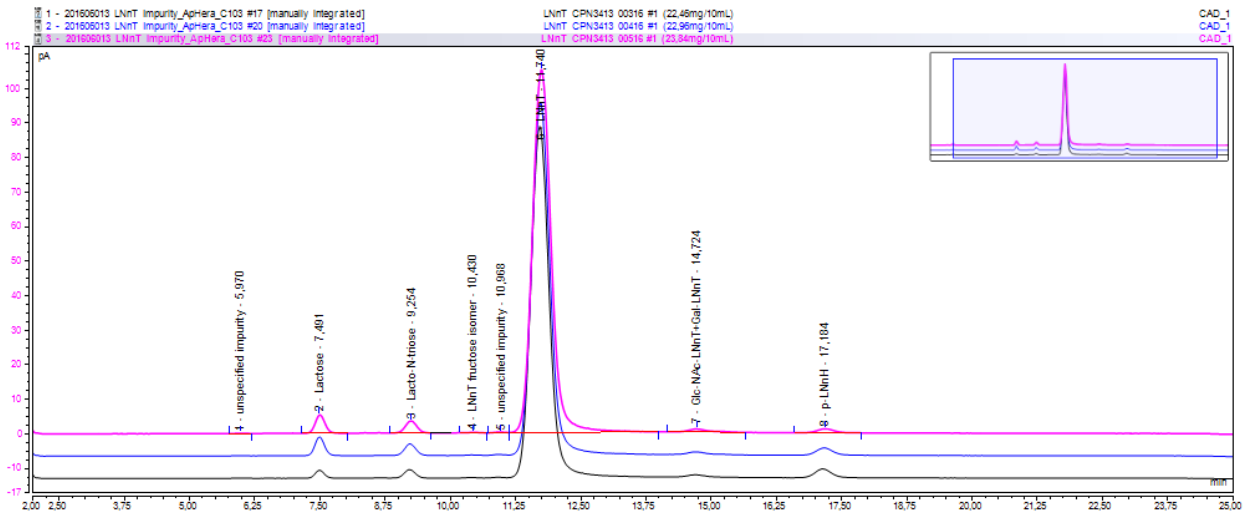


Figure B.5.1.2-2 Impurity Profile for Three Batches of LNnT, as Measured by High Performance Liquid Chromatography with a Corona Charged Aerosol Detector



Specifications for these carbohydrate-type compounds have been established and in-process controls (*i.e.*, for temperature and pH) and purification steps (*i.e.*, chromatography) have been included in the manufacturing process in order to minimise the formation of these substances. The results of batch analyses demonstrate that concentrations of each compound do not exceed 2% of the total ingredient, and at such low levels of concentration, they are not anticipated to have any significant effect on the safety or nutritive value of the LNnT ingredient produced by Glycom.

B.5.2 Manufacturing Impurities and Contaminants

B.5.2.1 Absence of Amino Acids and Biogenic Amines

Both 2'-FL and LNnT are harvested from the fermentation medium without disruption of the production strain; however, it is noted that batches of the ingredients also have been analysed for secondary metabolites and cellular components that may originate from the fermentation medium. Analyses of the ingredients for biogenic amines (*e.g.*, histamine, tyramine, spermidine, cadaverine and putrescine), and amino acids and their metabolites [*e.g.*, glutamic acid, gamma-aminobutyric acid (GABA)] did not identify detectable levels of these contaminants in either ingredient.

B.5.2.2 Absence of Microbial Endotoxins and Residual Proteins

Internal specifications for lipopolysaccharides (*i.e.*, endotoxins) originating from the fermentation organism have been established as an additional quality control point to ensure that microbial endotoxins are efficiently removed and/or not introduced during the production process of 2'-FL and LNnT. The endotoxin specifications are assayed using the *Limulus* amebocyte lysate kinetic chromogenic assay described in the European Pharmacopoeia. Batch analyses of 2'-FL and LNnT demonstrate compliance to the endotoxins specifications (see Tables B.6.2-1 and B.6.2-2).

The absence of residual proteins is confirmed by an adaptation of the Bradford protein method, which has been validated to detect and quantify residual protein down to a level of 0.0005 and 0.0017%, respectively. Protein levels were consistently below the quantitation limit for all batches and 2'-FL and LNnT tested (see Tables B.6.3-1 and B.6.3-2).

B.5.2.3 Absence of Production Organism

In the manufacturing of both 2'-FL and LNnT, the production microorganism is efficiently removed by the ultrafiltration step which is applied directly after fermentation during USP. Additionally, during DSP, various sequential purification processes are applied to ensure the absence of microbiological contamination. The overall purification process comprises of the following steps:

1. Ultrafiltration/diafiltration
2. Nanofiltration
3. Ion removal
4. Decolouration (*e.g.*, active charcoal filtration)
5. Microfiltration
6. Chromatography (for the purification of LNnT only)
7. Pre-concentration
8. Crystallisation
9. Solid-liquid-separation (*e.g.*, filtration)
10. Washing
11. Drying.

The absence of the microorganisms in the 2'-FL and LNnT ingredients is demonstrated by microbial testing for *Enterobacteriaceae* during batch analyses according to internationally-recognised methods (ISO 21528-1:2004, MSZ ISO 21528-2:2007). The ISO 21528-1:2004 method includes a pre-enrichment step to allow for resuscitation of the microorganism before enrichment. Additionally, the ISO 7251:2005 method for analysis of *E. coli* also has been applied to production batches of the ingredient during QC testing to further corroborate the absence of *Enterobacteriaceae*.

Finally, the absence of the production organism in the ingredient is also supported by the analysis of residual DNA in batches of the final ingredients. As shown in Table B.5.2.3-1, the absences of residual DNA from the production organism for 2'-FL and LNnT are each confirmed by 3 different validated quantitative polymerase chain reaction (qPCR) methods. The qPCR methods for 2'-FL target short subsequences of the marker genes *ampR* and *tetR* (located on the high-copy plasmids) and a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. For LNnT, the qPCR methods target subsequences of the inserted genes *galT* and *lgtA* as well as the same subsequence of the 23S ribosomal subunit of *E. coli*. Analysis of 4 batches of 2'-FL and LNnT demonstrate no quantifiable levels of residual DNA (limit of quantification = 4 ppb) present in the final ingredient.

Table B.5.2.3-1 Levels of Residual DNA in 4 Batches of 2'-FL and LNnT

Parameter	Specification	Average Batch Result	Lot 00215	Lot 00315	Lot 00415	Lot 00515
2'-FL						
Residual DNA by qPCR (AMP assay)	<LOQ[a]	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Residual DNA by qPCR (TET assay)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Residual DNA by qPCR (EC23S assay)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
LNnT						
Residual DNA by qPCR (*galT* assay)	<LOQ[a]	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Residual DNA by qPCR (*lgtA* assay)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Residual DNA by qPCR (EC23S assay)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ

2'-FL = 2'-*O*-fucosyllactose; AMP = adenosine monophosphate; DNA = deoxyribonucleic acid; LNnT = lacto-*N*-neotetraose; LOQ = limit of quantitation; qPCR = quantitative polymerase chain reaction; TET = tetracycline.
[a] LOQ = 4 µg/kg (parts per billion)

B.5.2.4 Residual Minerals from the Fermentation Medium

Although some residual amounts of minerals may remain in the 2'-FL ingredient, as a result of it being carried over from the fermentation medium, this is not expected to contribute significantly to the overall mineral content in finished food products (*i.e.*, infant formula, follow-on formula, and formulated supplementary foods for young children). As an example, the approximate contribution of minerals from its residual presence in the 2'-FL ingredient is presented in the table below, as compared against the mineral content requirements established in Schedule 29 of the Food Standards Code for finished infant formula products. The amount of minerals in the finished infant formula product, as contributed by its residual presence in the 2'-FL ingredient, is considered to be minimal. It should also be highlighted that the finished infant formula product will conform to the composition requirements as established in Standard 2.9.1 and Schedule 29 of the Food Standards Code.

Table B.5.2.4-1 Mineral and Lactose Contribution of 2'-FL to Infant Formula

Nutrient	Mean Results from 4 Lots of 2'-FL Produced by Fermentation (mg/kg)	Nutrient Value Provided by a Use-Level of 1.2 g/L 2'-FL[a] (mg/100 kJ)	Mineral Requirements in Infant Formula (Schedule 29) (mg/100 kJ)
Phosphate	3 (as orthophosphate)	0.0001	6 to 25 (as phosphorus)
Chloride	≤40	≤0.0019	12 to 35
Sodium (Na)	232	0.0111	5 to 15
Potassium (K)	163	0.0078	20 to 50
Magnesium (Mg)	826	0.0396	1.2 to 4
Calcium (Ca)	648	0.0311	12 to 33[b]
Iron (Fe)	3.5	0.0002	0.2 to 0.5
Zinc (Zn)	<2	<0.0001	0.12 to 0.43
Copper (Cu)	<1	<0.00005	0.014 to 0.043
Manganese (Mn)	1.25	0.0001	0.00024 to 0.0024

[a] In accordance with Standard 2.9.1 of the Food Standards Code (2.9.1–9), it was assumed that 1 L of formula contained a minimum of 2,500 kJ of energy.
[b] No maximum level for calcium that is required to be met for infant formula products is provided for under Schedule 29 (S29–9) of the Food Standards Code. However, under S29 – 10 (Guidelines for infant formula products), the recommended maximum level for calcium in infant formula products is 33 mg/100 kJ.

For LNnT, there are no appreciable levels of minerals carried over from the production process into the final ingredient. The results of trace element analyses for LNnT are presented in Table B.5.2.4-2 below.

Table B.5.2.4-2 Presence of Trace Elements in LNnT

Trace Element	LNnT Internal Specification	Batch Results			
		2547750801	2547750901	2547750902	2547750903
Phosphorus (as orthophosphate) (%)	Max. 0.5	<0.0010	<0.0010	<0.0010	<0.0010
Chloride (Cl) (%)	Max. 0.1	<0.0010	<0.0010	<0.0010	<0.0010
Sodium (Na) (mg/kg)	Max. 5,000	<10	<10	<10	<10
Potassium (K) (mg/kg)	Max. 5,000	<10	<10	<10	<10
Magnesium (Mg) (mg/kg)	Max. 3,000	<10	<10	<10	<10
Calcium (Ca) (mg/kg)	Max. 1,000	<10	<10	<10	<10
Iron (Fe) (mg/kg)	Max. 20	3	<1	<1	<1
Zinc (Zn) (mg/kg)	Max. 20	0.3	0.2	0.2	0.4
Copper (Cu) (mg/kg)	Max. 20	<0.1	<0.1	<0.1	<0.1
Manganese (Mn) (mg/kg)	Max. 10	<0.1	<0.1	<0.1	<0.1
Aluminium (Al) (mg/kg)	Max. 10	<2	<2	<2	<2
Chromium (Cr) (mg/kg)	Max. 3.0	0.2	<0.1	<0.1	<0.1
Molybdenum (Mo) (mg/kg)	Max. 1.0	0.1	<0.1	<0.1	<0.1
Cobalt (Co) (mg/kg)	Max. 1.0	<0.1	<0.1	<0.1	<0.1
Arsenic (As) (mg/kg)	Max. 0.4	<0.1	<0.1	<0.1	<0.1
Cadmium (Cd) (mg/kg)	Max. 0.05	<0.01	<0.01	<0.01	<0.01
Mercury (Hg) (mg/kg)	Max. 0.2	<0.01	<0.01	<0.01	<0.01

LNnT = lacto-*N*-Neotetraose; Max. = maximum

B.6 Specification for the Identity and Purity of the Novel Food Ingredient

B.6.1 Product Specifications for 2'-FL

2'-FL is not currently covered by one of the specifications described in Schedule 3 (Identity and Purity) of the Code. As mentioned, 2'-FL has been accepted for use in the EU and the U.S., and the material that will be marketed in Australia/New Zealand will meet the same product specification as defined in these other jurisdictions. The established specification for Glycom's 2'-FL ingredient, along with the methods of analysis, are provided in Table B.6.1-1. All of the methods of analysis used are nationally or internationally-recognised; when such methods do not exist, they were developed internally by Glycom and validated. Details of the internal methods of analysis are provided in Appendix VI-a.

Table B.6.1-1 Product Specification for 2'-FL

Parameter	Specification	Method
Appearance	Powder or agglomerates	ISO 6658:2007
Colour	White to off white	ISO 6658:2007
Identification	RT of main component corresponds to RT of standard ± 3%	Glycom method HPLC-202-2C4-002
Assay (water free) for human-identical milk saccharides (HiMS)[a]	Not less than 96.0 w/w %	Glycom methods HPLC-202-2C4-002, HPLC-206-2C4-001 and HPAEC-206-001
Assay (water free) 2'-FL	Not less than 94.0 w/w %	Glycom method HPLC-202-2C4-002
D-Lactose	Not more than 3.0 w/w %	Glycom method HPLC-206-2C4-001
L-Fucose	Not more than 1.0 w/w %	Glycom method HPAEC-206-001
Difucosyllactose	Not more than 1.0 w/w %	Glycom method HPAEC-206-001
2'-Fucosyl-D-lactulose	Not more than 1.0 w/w %	Glycom method HPLC-206-2C4-001
pH (20°C, 5% solution)	3.2 to 5.0	Ph. Eur. 2.2.3
Water	Not more than 5.0 w/w %	Karl-Fischer (Ph. Eur. 2.5.32)

Table B.6.1-1 Product Specification for 2'-FL

Parameter	Specification	Method
Ash, sulphated	Not more than 1.5 w/w %	Ph. Eur. 6.7 04/2010:20414
Acetic acid (as free acid and/or sodium acetate)	Not more than 1.0 w/w %	Megazyme K-ACETRM 07/12
Residual proteins	Not more than 0.01 w/w %	Bradford Assay; Glycom method UV-001
Heavy metals		
Lead	Not more than 0.1 mg/kg	ICP-MS by EPA 6020A:2007
Microbiological Parameters		
Salmonella	Absent in 25 g	ISO 6579:2006
Total plate count	Not more than 500 CFU/g	ISO 4833-1:2014
Enterobacteriaceae	Absent in 10 g	ISO 21528-1:2004, ISO 21528-2:2007
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	ISO 11290-1:1996/A1:2005
Bacillus cereus	Not more than 50 CFU/g	ISO 7932:2005
Yeasts	Not more than 10 CFU/g	ISO 7954:1999
Moulds	Not more than 10 CFU/g	ISO 7954:1999
Residual endotoxins	Not more than 10 EU/mg	Eur. Ph. 2.6.14

2'-FL = 2'-*O*-fucosyllactose; CFU = colony forming units; Eur. Ph. = European Pharmacopeia; EU = endotoxin units; HPAEC = high-performance anion-exchange chromatography; HPLC = high performance liquid chromatography; ISO = International Organization for Standardization; RT = retention time.
[a] Human-identical milk saccharides (HiMS) is defined as the sum of 2'-FL, lactose, difucosyllactose, and fucose.

B.6.2 Product Specifications for LNnT

LNnT is not currently covered by one of the specifications described in Schedule 3 (Identity and Purity) of the Code. As mentioned, LNnT has been accepted for use in the EU and the U.S., and the material that will be marketed in Australia/New Zealand will meet the same product specification as defined in these other jurisdictions. The established specification for Glycom's LNnT ingredient, along with the methods of analysis, are provided in Table B.6.2-1. All of the methods of analysis used are nationally or internationally-recognised; when such methods do not exist, they were developed internally by Glycom and validated. Details of the internal methods of analysis are provided in Appendix VI-a.

Table B.6.2-1 Product Specifications for LNnT

Parameter	Specification	Method
Appearance	Powder or agglomerates	ISO 6658:2007
Colour	White to off white	ISO 6658:2007
Identification	RT of standard ± 3%	Glycom method HPLC-106-1C6-002
Assay (water free) for human-identical milk saccharides (HiMS)[a]	Not less than 95.0 w/w %	Glycom method HPLC-106-1C6-002
Assay (water free) Lacto-N-neotetraose	Not less than 92.0 w/w %	Glycom method HPLC-106-1C6-002
D-Lactose	Not more than 3.0 w/w %	Glycom method HPLC-106-1C6-002
Lacto-*N*-triose II	Not more than 3.0 w/w %	Glycom method HPLC-106-1C6-002
para-Lacto-*N*-neohexaose	Not more than 3.0 w/w %	Glycom method HPLC-106-1C6-002
LNnT fructose isomer	Not more than 1.0 w/w %	Glycom method HPLC-106-1C6-002
pH (20°C, 5 % solution)	4.0 – 7.0	Eur. Ph. 2.2.3
Water	Not more than 9.0 w/w %	Karl-Fischer (Ph. Eur. 2.5.12)
Ash, sulphated	Not more than 1.5 w/w %	Eur. Ph. 6.7 04/2010:20414
Methanol	Not more than 100 mg/kg	Glycom method GC-109-1C6-001
Residual proteins	Not more than 0.01 w/w %	Bradford Assay; Glycom method UV-001

Table B.6.2-1 Product Specifications for LNnT

Parameter	Specification	Method
Heavy metals		
Lead	Not more than 0.1 mg/kg	ICP-MS by EPA 6020A:2007
Microbiological Parameters		
Salmonella	Absent in 25 g	ISO 6579:2006
Total plate count	Not more than 500 CFU/g	ISO 4833-1:2014
Enterobacteriaceae	Absent in 10 g	ISO 21528-1:2004, ISO 21528-2:2007
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	ISO-TS 22964:2006
Listeria monocytogenes	Absent in 25 g	ISO 11290-1:1996/A1:2005
Bacillus cereus	Not more than 50 CFU/g	ISO 7932:2005
Yeasts	Not more than 10 CFU/g	ISO 7954:1999
Moulds	Not more than 10 CFU/g	ISO 7954:1999
Residual endotoxins	Not more than 10 EU/mg	Eur. Ph. 2.6.14

CFU = colony forming units; Eur. Ph. = European Pharmacopeia; EU = endotoxin units; GC-HS = headspace gas chromatography; HPLC = high performance liquid chromatography; ISO = International Organization for Standardization; LNnT = lacto-*N*-neotetraose; RT = retention time.

[a] Human-identical milk saccharides (HiMS) is defined as the sum of LNnT, lactose, lacto-*N*-triose II, and *para*-lacto-*N*-hexaose.

B.6.3 Batch Analysis

Batch analyses for 4 independent production batches of 2'-FL are provided in Table B.6.3-1 below. The results demonstrate that the final 2'-FL ingredient complies with the product specifications summarised in Table B.6.1-1 above. Certificates of Analyses for these batches, which include additional parameters initially monitored for reassurance, are provided in Appendix VII.

Table B.6.3-1 Batch Analyses for 2'-FL

Parameter	Specification	Manufacturing Batch Number			
		Batch 00215	Batch 00315	Batch 00415	Batch 00515
Appearance	Powder or agglomerates	Complies	Complies	Complies	Complies
Colour	White to off white	Complies	Complies	Complies	Complies
Identification	RT of standard ± 3%	Complies	Complies	Complies	Complies
Assay (water free) HiMS[a]	Not less than 96.0 w/w %	98.4%	99.1%	97.9%	99.3%
Assay (water free) 2'-FL	Not less than 94.0 w/w %	97.6%	98.4%	97.2%	98.4%
D-Lactose	Not more than 3.0 w/w %	0.6%	0.57%	0.57%	0.84%
L-Fucose	Not more than 1.0 w/w %	<0.03%	0.03%	0.03%	<0.03%
Difucosyllactose	Not more than 1.0 w/w %	0.21%	0.12%	0.05%	0.08%
2'-Fucosyl-D-lactulose	Not more than 1.0 w/w %	0.28%	0.23%	0.34%	0.32%
pH (20°C, 5% solution)	3.2 to 5.0	4.2	4.1	3.9	3.8
Water	Not more than 5.0 w/w %	0.47%	0.35%	0.38%	0.23%
Ash, sulphated	Not more than 1.5 w/w %	0.62%	0.69%	0.77%	0.77%
Acetic acid (as free acid and/or sodium acetate)	Not more than 1.0 w/w %	0.20%	0.25%	0.32%	0.41%
Residual proteins	Not more than 0.01 w/w %	<LOQ[b]	<LOD[b]	<LOQ[b]	<LOQ[b]
Heavy metals					
Lead	Not more than 0.1 mg/kg	<0.1	<0.1	<0.1	<0.1

Table B.6.3-1 Batch Analyses for 2'-FL

Parameter	Specification	Manufacturing Batch Number			
		Batch 00215	Batch 00315	Batch 00415	Batch 00515
Microbiological Parameters					
Salmonella	Absent in 25 g	Complies	Complies	Complies	Complies
Total plate count	Max. 500 CFU/g	Complies	Complies	Complies	Complies
Enterobacteriaceae	Absent in 10 g	Complies	Complies	Complies	Complies
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Complies	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25 g	Complies	Complies	Complies	Complies
Bacillus cereus	Max. 50 CFU/g	Complies	Complies	Complies	Complies
Yeasts	Max. 10 CFU/g	Complies	Complies	Complies	Complies
Moulds	Max. 10 CFU/g	Complies	Complies	Complies	Complies
Residual endotoxins	Not more than 10 EU/mg	Complies	Complies	Complies	Complies

2'-FL = 2'-*O*-Fucosyllactose; CFU = colony forming units; EU = endotoxin units; LOD = limit of detection; w/w = weight/weight.
[a] Human-identical milk saccharides (HiMS) is defined as the sum of 2'-FL, lactose, difucosyllactose, and fucose.
[b] The LOQ is 0.0017% (w/w), the LOD is 0.0005 % (w/w).

Batch analyses for 4 independent production batches of LNnT are provided in Table B.6.3-2 below. The results demonstrate that the final LNnT ingredient complies with the product specifications established above in Table B.6.2-1. Certificates of Analyses for these batches, which include additional parameters initially monitored for reassurance, are provided in Appendix VII.

Table B.6.3-2 Batch Analyses for LNnT

Parameter	Specification	Manufacturing Batch Number			
		5247750801	2547750901	2547750902	2547750903
Appearance	Powder or agglomerates	Complies	Complies	Complies	Complies
Colour	White to off white	Complies	Complies	Complies	Complies
Identification	RT of standard ± 3%	Complies	Complies	Complies	Complies
Assay (water free) HiMS[a]	Not less than 95.0 w/w %	98.5%	97.9%	97.2%	98.6%
Assay (water free) LNnT	Not less than 92.0 w/w %	97.1%	94.4%	95.0%	97.2%
D-Lactose	Not more than 3.0 w/w %	0.25%	0.66%	0.38%	0.21%
Lacto-*N*-triose II	Not more than 3.0 w/w %	1.01%	1.62%	0.65%	0.65%
para-Lacto-*N*-neohexaose	Not more than 3.0 w/w %	0.13%	0.95%	1.02%	0.45%
LNnT fructose isomer	Not more than 1.0 w/w %	0.03%	0.40%	0.41%	0.29%
pH (20 °C, 5 % solution)	4.0 to 7.0	5.3	5.8	5.4	6.0
Water	Not more than 9.0 w/w %	6.6%	8.0%	7.8%	7.6%
Ash, sulphated	Not more than 1.5 w/w %	<0.03%	<0.01%	0.03%	<0.01%
Methanol	Not more than 100 mg/kg	57 mg/kg	19 mg/kg	32 mg/kg	22 mg/kg
Residual proteins	Not more than 0.01 w/w %	<LOQ[b]	<LOD[b]	<LOQ[b]	<LOQ[b]
Heavy metals					
Lead	Not more than 0.1 mg/kg	<0.1 mg/kg	<0.1 mg/kg	<0.1 mg/kg	<0.1 mg/kg
Microbiological Parameters					
Salmonella	Absent in 25 g	Complies	Complies	Complies	Complies
Total plate count	Not more than 500 CFU/g	Complies	Complies	Complies	Complies
Enterobacteriaceae	Absent in 10 g	Complies	Complies	Complies	Complies
Cronobacter (Enterobacter) sakazakii	Absent in 10 g	Complies	Complies	Complies	Complies
Listeria monocytogenes	Absent in 25 g	Complies	Complies	Complies	Complies
Bacillus cereus	Not more than 50 CFU/g	Complies	Complies	Complies	Complies

Table B.6.3-2 Batch Analyses for LNnT

Parameter	Specification	Manufacturing Batch Number			
		5247750801	2547750901	2547750902	2547750903
Yeasts	Not more than 10 CFU/g	Complies	Complies	Complies	Complies
Moulds	Not more than 10 CFU/g	Complies	Complies	Complies	Complies
Residual endotoxins	Not more than 10 EU/mg	Complies	Complies	Complies	Complies

CFU = colony forming units; EU = endotoxin units; HiMS = human-identical milk saccharides; LNnT = lacto-*N*-neotetraose; RT = retention time.

[a] Human-identical milk saccharides (HiMS) is defined as the sum of LNnT, lactose, lacto-*N*-triose II, and para-lacto-*N*-hexaose.

[b] The LOQ is 0.0017% (w/w), the LOD is 0.0005% (w/w).

B.7 Stability of the Novel Food Ingredient

B.7.1 Bulk Stability

B.7.1.1 Real-Time Stability Studies for 2'-FL

The stability of crystalline 2'-FL has been established for 2'-FL produced by chemical synthesis, and current investigation for crystalline 2'-FL produced by fermentation has shown similar results to date. It has been confirmed that the crystalline 2'-FL from the fermentation production process possesses the identical crystal form as the crystalline 2'-FL from chemical synthesis. Therefore, data from both stability studies are presented as they apply to crystalline 2'-FL independent of its source.

The bulk stability of crystalline 2'-FL from chemical synthesis has been evaluated under several long-term studies conducted both in real-time conditions (25°C, 60% relative humidity) and accelerated conditions (40°C, 75% relative humidity). Representative results of the 5-year real-time stability study are presented in the table below. Overall, the results of this study indicate that there are no changes in organoleptic properties of 2'-FL, no appreciable degradation of 2'-FL, no changes in impurity profile, and no alterations in the microbiological quality of the ingredient following storage for up to 5 years under ambient storage conditions. 2'-FL was analysed by HPLC and water content was analysed by Karl Fischer titration at each time point.

The current shelf-life of crystalline 2'-FL has thus been established as 5 years when stored at ambient temperature, protected from humidity.

Table B.7.1.1-1 Results of the 5-Year Bulk Stability Study on Chemically Synthesised Crystalline 2'-FL

Parameter	Baseline	Sample Time (Months)			
		12	24	48	60
Purity					
Description	A	A	A	A	B
Water content (w/w%)	0.1	0.3	0.5	0.4	0.3
2'-FL Assay (w/w%) (corrected for water content)	99.9	99.0	99.9	96.8	97.4
Lactose (w/w%)	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Microbiological Quality					
Salmonella (/25 g)	Negative	Negative	Negative	Negative	Negative
Aerobic mesophilic total count (CFU/g)	10	<10	<10	<10	<10
Enterobacteriaceae (/10 g)	Negative	Negative	Negative	Negative	Negative
Cronobacter (Enterobacter) sakazakii (/10 g)	Negative	Negative	Negative	Negative	Negative

2'-FL = 2'-*O*-fucosyllactose; CFU = colony forming units.
Note: A: White powder. B: Slightly agglutinated powder.

Similarly, the stability of crystalline 2'-FL produced from fermentation, as described herein, is currently being investigated in an ongoing 5-year study, with interim results available to 18 months (see Table B.7.1.1-2 below). The results further confirm that the ingredient is stable when stored at ambient room temperature (25°C, 60% relative humidity) for at least 18 months.

Table B.7.1.1-2 Interim Results of the 5-Year Real-Time Stability Study on Crystalline 2'-FL Obtained by Fermentation

Parameter	Baseline	Sample Time (Months)			
		3	6	12	18
Physical Properties					
Colour	White	White	White	White	White
Appearance	Slightly agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder	Agglutinated powder
Purity					
2'-FL Assay, Water-free (w/w%)	98.1	96.0	97.1	98.0	97.7
Water content (w/w%)	0.60	0.60	0.58	0.51	0.40
Lactose (w/w%)	0.50	0.56	0.58	0.70	0.59
Fucose (w/w%)	Not tested	Not tested	< 0.1	< 0.1	< 0.1
Monoacetyl-2'-FL (w/w%)	0.27	0.27	0.28	0.30	0.28
Microbiological Quality					
Microbiological Quality	Meets spec	Not tested	Not tested	Meets spec	Not tested

2'-FL = 2'-*O*-fucosyllactose; spec = specifications.

B.7.1.2 Accelerated Stability Studies for 2'-FL

The stability of crystalline 2'-FL produced by chemical synthesis and fermentation has been evaluated in 2-year accelerated stability studies at 40°C and 75% relative humidity. No changes in any of the parameters assessed were observed in the 2-year accelerated stability study for 2'-FL produced by chemical synthesis. The interim results (up to 18 months) obtained for the 2'-FL produced by fermentation in the accelerated stability study are presented below in Table B.7.1.2-1-2. As with the real-time stability testing, no appreciable changes in organoleptic properties nor degradation of the ingredient or alterations in impurity profiles were observed.

Table B.7.1.2-1 Interim Results of the 2-Year Accelerated Stability Study on Crystalline 2'-FL Obtained by Fermentation

Parameter	Baseline	Sample Time (Months)			
		1	6	12	18
Physical Properties					
Colour	White	White	White	White	White
Appearance	Slightly agglutinated powder	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder	Agglutinated powder
Purity					
2'-FL Assay, Water-free (w/w%)	98.1	96.3	96.9	97.4	96.7
Water content (w/w%)	0.60	0.60	0.60	0.46	0.38
Lactose (w/w%)	0.50	0.71	0.60	0.72	0.62
Fucose (w/w%)	Not tested	Not tested	< 0.1	< 0.1	< 0.1
Monoacetyl-2'-FL (w/w%)	0.27	0.39	0.32	0.33	0.32
Microbiological Quality					
Microbiological Quality	Meets spec	Not tested	Meets spec	Meets spec	Not tested

2'-FL = 2'-*O*-fucosyllactose; spec = specifications.

B.7.1.3 Real-Time Stability Studies for LNnT

The stability of crystalline LNnT has been established for LNnT produced by chemical synthesis, and currently investigations for crystalline LNnT produced by fermentation have shown similar results to date. It has been confirmed that the crystalline LNnT from the fermentation production process possesses the identical crystal form as the crystalline LNnT from chemical synthesis. Therefore, the data from both stability studies are presented as they apply to crystalline LNnT independent of its source.

The bulk stability of crystalline LNnT from chemical synthesis has been comprehensively evaluated under several long-term studies conducted both in real-time conditions (25°C, 60% relative humidity) and accelerated conditions (40°C, 75% relative humidity). Representative results of the 5-year real-time stability study are presented in the table below. Overall, the results of this study indicate that there are no changes in organoleptic properties of LNnT, no appreciable degradation of LNnT, no changes in impurity profile, and no alterations in the microbiological quality of the ingredient following storage for up to 5 years under ambient storage conditions. LNnT was analysed by HPLC and water content was analysed by Karl Fischer titration at each time point.

The current shelf-life of crystalline LNnT has thus been established as 5 years when stored at ambient temperature, protected from humidity.

Table B.7.1.3-1 Results of the 5-Year Bulk Stability Study on Chemically-Synthesised Crystalline LNnT

Parameter	Baseline	Sample Time (Months)			
		12	24	48	60
Purity					
Sensory	White powder	White powder	White powder	White powder	Ivory white powder
Water content (w/w%)	1.8	2.9	4.3	4.0	2.3
LNnT Assay (w/w%) (corrected for water content)	98.9	99.4	97.6	98.1	99.9
Microbiological Quality					
Salmonella (/25 g)	Negative	Negative	Negative	Negative (in 10 g)	Negative
Aerobic mesophilic total count (CFU/g)	10	<10	<10	<10	<10
Enterobacteriaceae (/10 g)	Negative	Negative	Negative	Negative	Negative
Cronobacter (Enterobacter) sakazakii (/10 g)	Negative	Negative	Negative	Negative	Negative

CFU = colony forming units; LNnT = lacto-*N*-neotetraose.

Similarly, the stability of crystalline LNnT produced from fermentation, as described herein, is currently being investigated in an ongoing 5-year study, with interim results available to 12 months (see table below). The results further confirm that the ingredient is stable when stored at ambient room temperature (25°C, 60% relative humidity) for at least 12 months.

Table B.7.1.3-2 Interim Results of the 5-Year Bulk Stability Study on LNnT Obtained by Fermentation

Parameter	Baseline	Sample Time (Months)			
		3	6	9	12
Physical Properties					
Colour	White	White	White	White	White
Appearance	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder	Agglutinated powder	Agglutinated powder
Purity					
LNnT Assay, Water-free (w/w%)	97.6	97.5	97.2	97.2	96.8
Water content (w/w%)	8.40	9.40	9.32	9.13	8.90
Lactose (w/w%)	0.46	0.39	0.37	0.40	0.38
Lacto-N-triose (w/w%)	0.68	0.65	0.66	0.69	0.71
LNnT fructose isomer (w/w%)	0.39	0.34	0.36	0.35	0.39
Microbiological Quality					
Microbiological Quality	Meets spec	Not tested	Not tested	Not tested	Meets spec

LNnT = lacto-*N*-neotetraose; spec = specifications.

B.7.1.4 Accelerated Stability Studies for LNnT

The stability of crystalline LNnT produced by chemical synthesis and fermentation has been evaluated in a 2-year accelerated stability studies at 40°C and 75% relative humidity. No changes in any of the parameters assessed were observed in the 2-year accelerated stability study for LNnT produced by chemical synthesis. The interim results (up to 12 months) obtained for the LNnT produced by fermentation in the accelerated stability study are presented in the Table below. As with the real-time stability testing, no appreciable changes in organoleptic properties nor degradation of the ingredient or alterations in impurity profiles were observed.

Table B.7.1.4-1 Interim Results of the 2-Year Accelerated Stability Study on LNnT Obtained by Fermentation

Parameter	Baseline	Sample Time (Months)			
		3	6	9	12
Physical Properties					
Colour	White	White	White	White	White
Appearance	Slightly agglutinated powder	Slightly agglutinated powder	Agglutinated powder	Agglutinated powder	Agglutinated powder
Purity					
LNnT Assay, Water-free (w/w%)	97.6	97.2	97.0	97.0	97.1
Water content (w/w%)	8.4	9.3	9.3	8.6	9.0
Lactose (w/w%)	0.46	0.41	0.40	0.41	0.40
Lacto-N-triose (w/w%)	0.68	0.67	0.71	0.72	0.73
LNnT fructose isomer (w/w%)	0.39	0.35	0.38	0.39	0.41
Microbiological Quality					
Microbiological Quality	Meets spec	Not tested	Meets spec	Not tested	Meets spec

LNnT = lacto-*N*-neotetraose; spec = specifications.

B.7.2 Stability Under the Intended Conditions of Use

B.7.2.1 Stability of 2'-FL and LNnT in Powdered Infant Formula

The stability of 2'-FL and LNnT in infant formula has been investigated following long-term storage. The infant formula powder tested was a whey-based starter formula (see Table B.7.2.1-1 for composition). In addition to 2'-FL and LNnT, the infant formula contained long chain polyunsaturated fatty acids (LC-PUFA), as well as vitamins and minerals at concentrations intended to provide complete nutrition for infants from birth to 6 months of age. The composition of this infant formula powder is representative of commercial infant formulas on the market.

Table B.7.2.1-1 Composition of the Infant Formula Powder Used in the 3-Year Stability Study

Parameter	Content
Protein (Whey proteins/caseins)	1.889 g/100 kcal (71.6 %/28.4 %)
Carbohydrate	11.182 g/100 kcal
Fat	5.302 g/100 kcal
Dry matter	97.51 %
Moisture	2.49 %
Caloric density	66.9 kcal/100 mL

The infant formula powder samples were produced following a commercial infant formula production process in which 2'-FL (Batch L06112K) and LNnT (Batch L01032K) were added in the wet mixture together with other ingredients (salts, carbohydrates, proteins). 2'-FL was added at a target concentration of 0.90 g/100 g (dry matter), while LNnT was added at a target concentration of 0.45 g/100 g (dry matter). This level of addition for 2'-FL and LNnT is considered to be representative of the usage that could occur under the intended conditions of use. Following dissolution of the ingredients in water, the mixture was heat-treated at a temperature of 105°C for 5 seconds. Subsequent steps consisted of evaporation, homogenisation, and spray drying to produce a powdered product. Samples were stored in gassed (N_2/CO_2) tin cans (1 can per time and temperature point) at a temperature of 4, 20, 30, or 37°C. Samples were dissolved immediately prior to analysis at a

reconstitution rate of 129 g/L and 2'-FL content was measured at regular time points up to 900 days of storage.

As demonstrated in the figures below, no significant loss of 2'-FL or LNnT occurred when these ingredients are added to an infant formula powder prior to processing, and stored at temperatures of up to 37°C over a period of 900 days. 2'-FL is therefore stable under the intended conditions of use in powdered infant formula under the recommended storage conditions and when subjected to higher storage temperatures (of up to 37°C) for prolonged periods.

Figure B.7.2.1-1 Stability of 2'-FL in a Commercially Representative Infant Formula Following Storage for up to 900 Days at Various Temperatures (Reconstituted at 129 g/L)

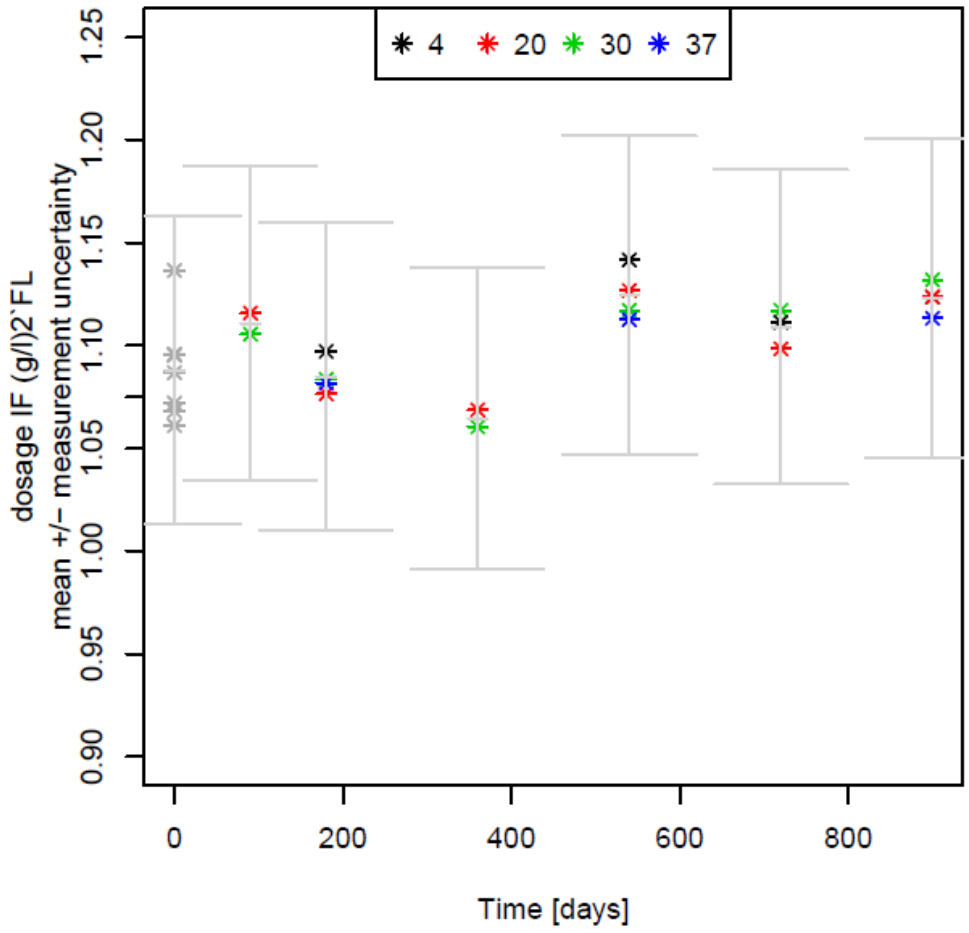
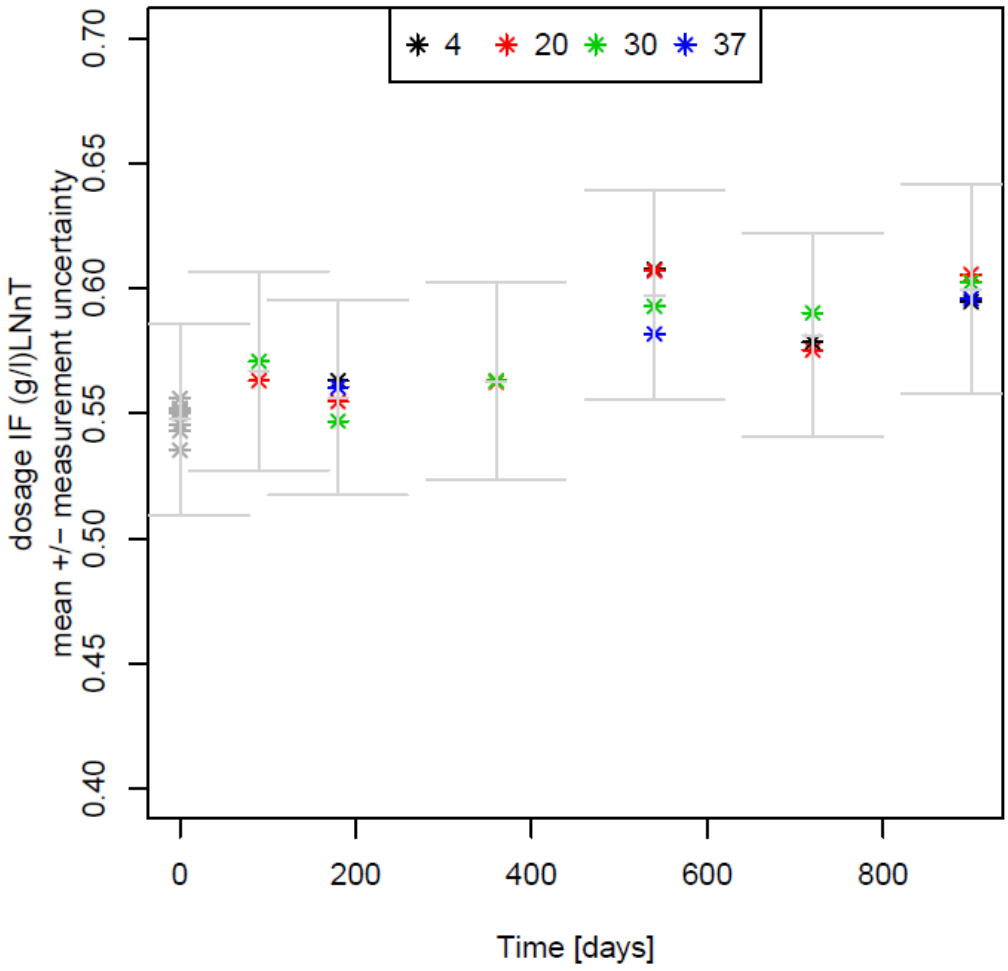


Figure B.7.2.1-2 Stability of LNnT in a Commercially Representative Infant Formula Following Storage for Up to 900 Days at Various Temperatures (Reconstituted at 129 g/L)



B.7.2.2 Stability of 2'-FL and LNnT in Other Food Matrices

It has been demonstrated that 2'-FL and LNnT are stable in other food products, including liquid matrices. 2'-FL and LNnT have been individually added to various food products, including yogurts, ready-to-drink flavoured milk, and citrus fruit beverages, following which their contents have been assessed using the same HPLC method with fluorescent detection as applied in the analysis of these HiMOs in infant formula. These stability studies were conducted using formulations representative of commercial food products on the market, and they were subjected to the processing (*i.e.*, pasteurisation and/or ultra-high-temperature heating) and storage conditions (*e.g.*, temperature) that would normally be applied to such products.

These studies demonstrate that there was no loss of 2'-FL or LNnT in yogurt, a citrus fruit drink, or ready-to drink chocolate-flavoured milk, at each time point tested, when compared to the initial concentrations. Pasteurisation and/or ultra-high temperature heating did not affect the stability of 2'-FL and LNnT in these foods. Therefore, 2'-FL and LNnT were demonstrated to be stable in liquid matrices, including yoghurt, citrus fruit drinks, and ready-to-drink chocolate-flavoured milk (see GRN 546 and 547 for analytical results; U.S. FDA, 2015a,c).

B.8 Analytical Method for the Detection of the Novel Food Ingredient in Foods

The presence of 2'-FL and LNnT in infant formula, follow-on formula, and formulated supplementary foods for young children can be detected, differentiated from other oligosaccharides, and reliably quantified using a derivatisation step prior to HPLC with fluorescent detection. Details of the method and its validation are provided in Appendix VI-b.

C. INFORMATION ON THE SAFETY OF THE NOVEL FOOD

Glycom's 2'-FL and LNnT ingredients belong to the following category of novel food: (IV) single chemical entities. The data to support the safety of these ingredients under their proposed conditions of use is described in this Section. Specifically, this Section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel Foods), Guideline 3.6.2 (Special Purpose Food – Infant Formula Products), and Guideline 3.6.3 (Special Purpose Foods – Other Foods) of the Food Standards Australia New Zealand Application Handbook (FSANZ, 2016). The corresponding Sections of this Application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	**Required Information Described in the Guideline**	**Section(s) of the Application where this is Addressed**
Guideline 3.5.2 – Novel Foods	***C.4 Single chemical entities and Dietary macro-components***	
	C.4.1 Information on the toxicokinetics and metabolism of the single chemical entity and, where appropriate, its degradation products and major metabolites	Section C.2
	C.4.2 Information from studies in animals or humans that is relevant to the toxicity of the single chemical entity and, where appropriate, its degradation products and major metabolites	Sections C.1, C.3, C.4, C.5, C.6
	C.4.3 Safety assessment reports prepared by international agencies or other national government agencies	Section C.7
Guideline 3.6.2 – Special Purpose Food (Infant Formula Products)	***A. Information related to composition***	
	A.3 Specific information requirements for the nutritional safety, tolerance and efficacy of the proposed compositional change (specifically subsection A.3.1)	
	b) Nutritional safety and tolerance of the proposed compositional change	Section C.1 to C.8
Guideline 3.6.3 – Special Purpose Food (Other foods)	***A. Information related to general compositional requirements***	
	A.3 Information related to the safety of the proposed compositional change	Section C.1 to C.8
Guideline 3.3.2 – Processing Aids	***C.2 Information on the potential toxicity of the enzyme processing aid***	
	a) Information on the enzyme's prior history of human consumption and its similarity to proteins with a history of safe human consumption b) Information on any significant similarity between the amino acid sequence of the enzyme and that of known protein toxins.	Section C.6
	C.3 Information on the potential allergenicity of the enzyme processing aid	Section C.6

C.1 History of Safe Consumption from Human Breast Milk

C.1.1 Human Milk Oligosaccharides

Human milk contains as its third largest solid component a fraction consisting of a complex family of structurally-related oligosaccharides (Kuhn, 1952; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013). These oligosaccharides are referred to as HMOs because they were first discovered in human breast milk (Malpress and Hytten, 1958), and they occur in human milk at much higher concentrations than in any other mammalian milk (Urashima *et al.*, 2001). HMOs consist of a lactose core, to which one or several of the 4 monosaccharides *N*-acetyl-D-glucose, D-galactose, L-fucose and/or *N*-acetyl-D-neuraminic acid ("sialic acid") are attached in specific connectivity and linkages. HMOs, including 2'-FL and LNnT, are biosynthesised in their free form exclusively in the lactating mammary gland by specific enzymes. Although HMOs are diverse in structure, they can be categorised into 3 main classes: neutral core HMOs (containing the aminosugar GlcNAc), neutral fucosylated HMOs (containing fucose), and acidic HMO (containing sialic acid).

The highest concentrations of HMOs occur in human colostrum (20 to 25 g/L), and concentrations between 5 to 20 g/L occur in mature human milk (Bode, 2012). In contrast, bovine milk contains approximately 20 times lower concentrations of a far less complex oligosaccharide mixture (Tao *et al.*, 2009; Aldredge *et al.*, 2013; Urashima *et al.*, 2013), which does not contain fucosylated oligosaccharides at any appreciable level (Gopal and Gill, 2000; Aldredge *et al.*, 2013). The respective composition of each mammalian milk oligosaccharide fraction allows interesting insights into evolutionary aspects of lactation (Urashima *et al.*, 2012).

C.1.2 Levels of 2'-FL in Human Breast Milk

The concentration of 2'-FL in human milk has been measured and reported to date in at least 28 independent publications. The following table summarises the levels of 2'-FL that have been reported in breast milk across these various studies. Additional details of these studies are available in Appendix III-b.

The data demonstrate that 2'-FL is, on average, the most abundant HMO of pooled human milk. It is worth mentioning that approximately 20% of women do not express the α-1,2-fucosyltransferase enzyme in their mammary glands (termed "non-secretors") (Castanys-Muñoz *et al.*, 2013; Austin *et al.*, 2016). This enzyme is responsible for fucosylating oligosaccharides and lactose at the 2'-*O*-position, resulting in the production of other HMOs, including 2'-FL. A further discussion of the secretor phenotype is provided in Section E and Appendix III-a, including data from observational studies that suggest that infants from Secretor mothers have various health benefits as compared to infants from non-Secretor mothers.

Table C.1.2-1 2'-FL Concentration in Human Milk after Full-Term Birth

Lactation Time	Key Findings	References
Pooled milk		
Days 1-4 ("colostrum")	**Reported Range: 1.0 to 8.4 g/L** Average: 3.2 g/L	Erney *et al.*, 2000; Morrow *et al.*, 2004; Asakuma *et al.*, 2008; Spevacek *et al.*, 2015
Days 5-14 ("transitional milk")	**Reported Range: 2.1 to 2.8 g/L** Average: 2.5 g/L	Erney *et al.*, 2000; Spevacek *et al.*, 2015; Austin *et al.*, 2016
Days 10-60 ("mature milk")	**Reported Range: 0.7 to 3.9 g/L** Average: 2.2 g/L	Chaturvedi *et al.*, 1997, 2001; Erney *et al.*, 2000, 2001; Musumeci *et al.*, 2006; Spevacek *et al.*, 2015; Austin *et al.*, 2016 ; McGuire *et al.*, 2017
After 2 months ("mature milk")	**Reported Range: 0.7 to 3.4 g/L** Average: 1.9 g/L	Erney *et al.*, 2000; Asakuma *et al.*, 2011; Smilowitz *et al.*, 2013; Austin *et al.*, 2016 ; McGuire *et al.*, 2017

Table C.1.2-1 2'-FL Concentration in Human Milk after Full-Term Birth

Lactation Time	Key Findings	References
Secretor milk		
Days 1-4 ("colostrum")	**Reported Range: 3.9 to 4.1 g/L** Average: 4.0 g/L	Coppa *et al.*, 1999; Leo *et al.*, 2009; Kunz *et al.*, 2017
Days 5-14 ("transitional milk")	**Reported Range: 3.0 to 3.6 g/L** Average: 3.3 g/L	Coppa *et al.*, 1999; Kunz *et al.*, 2017
Days 10-60 ("mature milk")	**Reported Range: 1.0 to 7.8 g/L** Average: 3.0 g/L	Coppa *et al.*, 1999, 2011; Leo *et al.*, 2009; Galeotti *et al.*, 2012, 2014; Bao *et al.*, 2013; Hong *et al.*, 2014; Olivares *et al.*, 2015; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a; McGuire *et al.*, 2017
After 2 months ("mature milk")	**Reported Range: 1.0 to 3.6 g/L** Average: 2.4 g/L	Thurl *et al.*, 1996; Coppa *et al.*, 1999; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a ; McGuire *et al.*, 2017

2'-FL = 2'-*O*-fucosyllactose.

The **average** levels in pooled milk are highest in colostrum (3.2 g/L), followed by transitional milk (2.5 g/L) and continue to decline slowly in mature milk (2.2 g/L) and mature milk from a lactation stage later than 2 months (1.9 g/L). In the context of relative abundance, 2'-FL ranks first with approximately 15 to 20 w/w % (corresponding to 24 to 30 mol %) of the total HMO biomass (Castanys-Muñoz *et al.*, 2013). In milk from Secretor mothers, the corresponding levels are significantly higher, with average levels reported at 4.0 g/L in colostrum, 3.3 g/L in transitional milk, 3.0 g/L in mature milk and 2.4 g/L in mature milk from a lactation stage later than 2 months. It is notable that 2'-FL concentrations between different mothers can be highly variable, with reported levels reaching beyond 5 g/L in some cases.

Several studies have also investigated the regional (ethnic) dependency of the 2'-FL concentration of milk and reveal that the correlation to the Secretor frequency within a population is predictive. There are negligible differences of average 2'-FL concentrations (2.2 to 2.4 g/L) between Asia, China, Europe and the U.S., regions which all possess Secretor frequencies between 70 and 80% (see table below). In Mexico, Peru, and the Hispanic populations of the U.S., where the Secretor frequency is reported to reach nearly 100%, the average concentration of 2'-FL is highest with 3.2 to 3.4 g/L.

Table C.1.2-1 2'-FL Concentration in Mature Human Milk (Lactation Days 10 to 60) by Global Regions

Region	Secretor frequency	Key findings	References
Latin America (Mexico, Peru)	~ 98-100 %	**Average: 3.2 g/L**	Erney *et al.*, 2000; Morrow *et al.*, 2004; McGuire *et al.*, 2017
Asia	~ 80 %	**Average: 2.3 g/L**	Erney *et al.*, 2000
China	~ 80 %	**Average: 2.3 g/L**	Austin *et al.*, 2016
Europe	~ 76-80 %	**Average: 2.4 g/L**	Erney *et al.*, 2000; McGuire *et al.*, 2017
U.S.	~ 68-75 %	**Average: 2.2 g/L**	Erney *et al.*, 2000; Chaturvedi *et al.*, 2001; Spevacek *et al.*, 2015; McGuire *et al.*, 2017
U.S. (Hispanic)	~ 95 %	**Average: 3.4 g/L**	McGuire et al., 2017
Africa	~ 65-85 %	**Average: 1.3 g/L**	McGuire et al., 2017
World	~ 80%	**Global average: 2.4 g/L** **Reported Range: 0 to 7.8 g/L**	Ferrer-Admetlla *et al.*, 2009

2'-FL = 2'-*O*-fucosyllactose; U.S. = United States.

C.1.3 Levels of LNnT in Human Breast Milk

The concentration of LNnT in human milk has been measured and reported to date in at least 26 independent publications. The following table summarises the levels of LNnT that have been reported in breast milk across these various studies. Additional details of these studies are available in Appendix III-b.

Table C.1.3-1 LNnT Concentration in Human Milk after Full-Term Birth

Lactation time	Key findings	References
Pooled milk		
Days 1-4 ("colostrum")	**Reported Range: 0.21 to 0.49 g/L** Average: 0.34 g/L Outlier: 2.04 g/L (Coppa)	Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Asakuma *et al.*, 2008; Thurl *et al.*, 2010; Bao *et al.*, 2013; Spevacek *et al.*, 2015; Kunz *et al.*, 2017
Days 5-14 ("transitional milk")	**Reported Range: 0.15 to 0.55 g/L** Average: 0.32 g/L Outlier: 1.83 g/L (Coppa)	Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Leo *et al.*, 2009; Leo *et al.*, 2010; Spevacek *et al.*, 2015; Austin *et al.*, 2016; Kunz *et al.*, 2017
Days 10-60 ("mature milk")	**Reported Range: 0.09 to 1.08 g/L** Average: 0.31 g/L Outliers: 0.95 to 4.1 g/L (Coppa, Galeotti)	Chaturvedi *et al.*, 1997; Coppa *et al.*, 1999; Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Leo *et al.*, 2009; Thurl *et al.*, 2010; Bao *et al.*, 2013; Galeotti *et al.*, 2014; Hong *et al.*, 2014; Spevacek *et al.*, 2015; Austin *et al.*, 2016; Kunz *et al.*, 2017; McGuire et al., 2017; Sprenger *et al.*, 2017a
After 2 months ("mature milk")	**Reported Range: 0.04 to 1.08 g/L** Average: 0.28 g/L Outliers: 1.37 g/L (Coppa)	Thurl *et al.*, 1996; Coppa *et al.*, 1999; Nakhla *et al.*, 1999; Erney *et al.*, 2000; Chaturvedi *et al.*, 2001; Sumiyoshi *et al.*, 2003; Leo *et al.*, 2010; Asakuma *et al.*, 2011; Smilowitz *et al.*, 2013; Austin *et al.*, 2016; Kunz *et al.*, 2017; McGuire et al., 2017; Sprenger *et al.*, 2017a
Secretor milk		
Days 1-30	**Average: 0.30 g/L** Reported Range: 0.24 to 0.36 g/L Outliers: 2.57 g/L (Galeotti)	Thurl *et al.*, 2010; Galeotti *et al.*, 2012; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a
Non-secretor milk		
Days 1-30	**Average: 0.19 g/L** Reported Range: 0.11 to 0.25 g/L Outliers: 3.53 g/L (Galeotti)	Thurl *et al.*, 2010; Galeotti *et al.*, 2012; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a

LNnT = lacto-*N*-neotetraose.

It is important to recognise that LNnT is present in the milk of all mothers. The average levels in pooled milk are highest in colostrum (0.34 g/L), followed by transitional milk (0.32 g/L) and continue to decline slowly in mature milk (0.31 g/L) and mature milk from a lactation stage later than 2 months (0.28 g/L). The reported ranges are between 0.04 and 1.08 g/L. Some studies find significantly higher levels of LNnT in breast-milk, but as these studies are all connected to the same group of investigators and are inconsistent with numerous reports by a several independent researchers, applying a set of different analytical methods, they have been considered as outliers for this analysis (Coppa *et al.*, 1999; Gabrielli *et al.*, 2011; Galeotti *et al.*, 2012, 2014).

In milk from Secretor mothers, the corresponding LNnT levels (0.30 g/L) are consistently higher than in the milk of non-Secretor mothers (0.19 g/L), as confirmed by four independent studies (Thurl *et al.*, 2010; Hong *et al.*, 2014; Kunz *et al.*, 2017; Sprenger *et al.*, 2017a) and suggesting a possible co-regulation of Secretor function and LNnT expression.

Several studies have also investigated the regional (ethnic) dependency of the LNnT concentration of milk. The average LNnT concentrations are comparable across Latin America, Asia, Europe, U.S. and Samoa (0.25 to 0.33 g/L). The reported levels for China appear to be lower (0.15 g/L), but are based on a single study to date. Although the African populations appear to possess higher levels of LNnT in their milk (0.7 g/L), this data is also based on a single study. Therefore, it is possible that the reported low

and high extremes may be study-biased, rather than real differences. The most parsimonious conclusion is that there is a wide variation between individual mothers that covers ranges up to more than 1 g/L of LNnT.

Table C.1.2-2 LNnT Concentration in Mature Human Milk (Lactation Days 1 to 60) by Global Regions

Region	Key Findings	References
Latin America (Mexico, Peru)	**Average: 0.33 g/L**	Chaturvedi *et al.*, 1997; Erney *et al.*, 2000; McGuire et al., 2017
Asia	**Average: 0.25 g/L**	Erney *et al.*, 2000; Sumiyoshi *et al.*, 2003; Asakuma *et al.*, 2008, 2011; Sprenger *et al.*, 2017a
China	**Average: 0.15 g/L**	Austin *et al.*, 2016
Europe	**Average: 0.32 g/L**	Erney *et al.*, 2000; Thurl *et al.*, 2010; Kunz *et al.*, 2017; McGuire *et al.*, 2017
U.S.	**Average: 0.33 g/L**	Erney *et al.*, 2000; Bao *et al.*, 2013; Hong *et al.*, 2014; Spevacek *et al.*, 2015; McGuire *et al.*, 2017
Africa	**Average: 0.70 g/L**	McGuire *et al.*, 2017
World	**Global Average: 0.34 g/L** **Reported Ranges: 0.04 to 1.08 g/L**	

LNnT = lacto-*N*-neotetraose; U.S. = United States.

C.2 Metabolic Fate

As mentioned, analytical data have demonstrated that Glycom's 2'-FL and LNnT ingredients are structurally and chemically identical to the 2'-FL and LNnT that are present in human breast milk (see Appendix IV). Therefore, Glycom's 2'-FL and LNnT ingredients will be subjected to the same metabolic pathways as their naturally occurring counterparts in human breast milk.

HMOs, including 2'-FL and LNnT, are recognised to be highly resistant to hydrolysis by digestive enzymes (Brand-Miller *et al.*, 1995, 1998; Engfer *et al.*, 2000; Gnoth *et al.*, 2000), and they reach the large intestine largely intact. While the majority of ingested HMOs pass through the gastrointestinal tract and are subject to fermentation by the microbiota in the large intestine (Brand-Miller *et al.*, 1995, 1998) or are excreted intact in the faeces (Chaturvedi *et al.*, 2001; Coppa *et al.*, 2001), there is a small fraction of HMOs that is absorbed into the systemic circulation. Mechanistically, Gnoth *et al.* (2001) have suggested that small quantities of HMOs are transported transcellularly across the intestinal epithelium by receptor-mediated transcytosis, and/or by paracellular means, and low quantities of HMOs have been detected unchanged in the urine of breast-fed infants (Rudloff *et al.*, 1996, 2012; Obermeier *et al.*, 1999; Chaturvedi *et al.*, 2001; Dotz *et al.*, 2014). The proportion of HMOs detected in the blood or urine are relatively small, with some reports of intact HMO concentrations in the blood and urine to be 0.1 and 4% of the concentrations measured in human milk (Goehring *et al.*, 2014). More recently, the work of Marriage *et al.* (2015) demonstrate that the relative absorption[4] of 2'-FL in the plasma is in the region of 0.02 and 0.07% for infants receiving formulae supplemented with 0.2 and 1.0 g 2'-FL/L, respectively.

The absorption and fermentability of specific HMOs have been demonstrated to be selective and age-dependent in human infants. The results of nano-high performance liquid chromatography - chip/time-of-flight mass spectrometry have shown that the chromatograms of human breast milk and that of the infant's faeces do differ in oligosaccharide profile, and levels of intact HMO structures are decreased in the faeces with increasing age (Davis *et al.*, 2016). It was also reported that intact 2'-FL was detected in

[4] Relative absorption was defined as the concentration of 2'-FL in plasma relative to the concentration in the formulae.

infant faeces at significantly lower concentrations than human breast milk, while LNnT was not detected at all, providing indirect evidence of their utilisation by the gut bacteria (Davis *et al.*, 2016).

Overall, it is expected that the majority of 2'-FL and LNnT consumed by infants will be transported intact to the large intestine and be subjected to fermentation by the intestinal microbiota populations, or otherwise become excreted intact in the faeces.

C.3 Toxicological Data for 2'-FL

C.3.1 Test Articles Used in the Toxicological Assessments

Glycom's 2'-FL ingredient obtained by fermentation has been the subject of comprehensive toxicological assessments, including a 90-day oral toxicity study, an *in vitro* bacterial reverse mutation assay, and an *in vitro* micronucleus assay. In addition, toxicological testing has also been conducted with chemically synthesised 2'-FL (also manufactured by Glycom), as well as a 2'-FL preparation obtained from fermentation that is manufactured by Jennewein.

A comparison of the test articles used in the various available toxicity studies is presented below in Table C.3.1-1. Glycom has conducted analysis demonstrating that the 2'-FL produced by fermentation is structurally identical to their purified 2'-FL obtained by chemical synthesis (see Appendix IV). The 2'-FL ingredient produced by Jennewein by fermentation is also a purified preparation consisting largely of 2'-FL. Therefore, the toxicological data obtained for these other 2'-FL preparations are considered to be relevant and are discussed herein.

Table C.3.1-1 Comparison of 2'-FL Test Articles Used in Toxicological Studies

Parameter	**2'-FL Preparation**		
	2'-FL as Described Herein	**Glycom A/S (Coulet *et al.*, 2014)**	**Jennewein (GRN 571)**
Test Article Purity	97.6%	99%	94.1%
Manufacture Process	Fermentation	Chemical synthesis	Fermentation
Production Organism	*Escherichia coli* K-12	NA	*E. coli* BL21
Specification			
Assay by HPLC	Min. 94.0%	Min. 95.0%	≥ 90%*
D-Lactose	Max. 3.0 w/w%	Max. 3.0 w/w%	≤ 5%*
L-Fucose	Max. 1.0 w/w%	Max. 1.0 w/w%	≤ 3%*
Difucosyllactose	Max. 1.0 w/w%	Max. 1.0 w/w%	≤ 5%*
2'-Fucosyl-D–lactulose	Max. 1.0 w/w%	Max. 0.6 w/w%	NS
Fucosyl galactose	NS	NS	≤ 3%*
Protein	0.01%	0.1%	≤ 100 ppm
Ash	Max. 1.5%	0.2%	≤ 5%
Preclinical Studies Conducted	Bacterial reverse mutation test *In vitro* micronucleus assay 90-day oral toxicity study	Bacterial reverse mutation test *In vitro* micronucleus assay *In vitro* mammalian cell gene mutation test 90-day oral toxicity study	90-day feeding study Ames test *In vivo* mouse micronucleus test Hanlon and Thorsrud (2014) Piglet feeding study

2'-FL = 2'-*O*-Fucosyllactose; HPLC = high-performance liquid chromatography; NA = not applicable; NS = not specified; ppm = parts per million; w/w = weight/weight.
* Percent of total carbohydrates by HPLC (area under the curve)

C.3.2 Mutagenicity/Genotoxicity

C.3.2.1 Studies Conducted with Glycom's 2'-FL Obtained by Fermentation

The mutagenicity of 2'-FL produced by fermentation (97.6% 2'-FL by assay), as described herein, was evaluated in a bacterial reverse mutation assay in *S.* Typhimurium strains TA98, TA100, TA1535, and TA1537 and in *E. coli* strain WP2uvrA in the presence or absence of metabolic activation (S9), using the plate incorporation and pre-incubation methods (Verspeek-Rip, 2015; Appendix VIII). The study was conducted in accordance with the Organization for Economic Cooperation and Development (OECD) principles of Good Laboratory Practice (GLP) and according to OECD Test Guideline No. 471 (OECD, 1997a, 1998a). The water vehicle served as a negative control for all strains. One of the following compounds was employed as a positive control for different strains in assays conducted in the absence of S9: 2-nitrofluorene (TA98, TA1537, pre-incubation assay), methylmethanesulfonate (TA100), sodium azide (TA1535), ICR-191 (TA1537, direct plate assay), 9-aminoacridine (TA1537), or 4-nitroquinoline n-oxide (WP2uvrA). For assays conducted in the presence of S9, 2-aminoathracene was employed as the positive control.

Using the plate incorporation method, bacterial strains were treated with 2'-FL at concentrations of 52, 164, 512, 1,600, or 5,000 µg/plate. For the pre-incubation method, bacterial strains were incubated with 2'-FL at concentrations of 492, 878, 1,568, 2,800, or 5,000 µg/plate. No cytotoxicity or precipitation was observed in any strain treated with 2'-FL in the presence or absence of S9. Treatment with 2'-FL did not result in a biologically significant increase in the number of revertant colonies compared with the negative control at any concentration in both experiments either in the presence or absence of S9. In contrast, positive control agents substantially induced an increase in the number of revertant colonies compared to the negative control. Thus, 2'-FL was determined to be non-mutagenic under the conditions of the bacterial reverse mutation assay in the presence or absence of exogenous metabolic activation at concentrations up to 5,000 µg/plate.

The genotoxicity of 2'-FL produced by fermentation (97.6% 2'-FL by assay) was further investigated in an *in vitro* micronucleus assay conducted in cultured peripheral human lymphocytes (Verbaan, 2015a; Appendix VIII). This study also was conducted in compliance with the OECD principles of GLP and according to OECD Test Guideline No. 487 (OECD, 1998a, 2014). Mitomycin C and cyclophosphamide were used as the positive controls in the absence and presence of S9, respectively, and water was used as the negative control. In the short-term exposure experiment, lymphocytes were incubated with 2'-FL at concentrations of 512, 1,600, or 2,000 µg/mL for 3 hours in the presence or absence of S9, following which the cells were rinsed and incubated for another 24 hours prior to scoring. In the long-term exposure experiment, cells were treated with 2'-FL at concentrations of 512, 1,600, or 2,000 µg/mL for 24 hours in the absence of S9. At least 1,000 binucleated cells and 1,000 mononucleated were scored for micronuclei under each treatment condition.

In both experiments, there were no signs of precipitation or cytotoxicity (as determined by the cytokinesis block proliferation index) observed in cells treated with 2'-FL at any concentration. No statistically or biologically significant increases in the frequency of mono- or bi-nucleated cells with micronuclei were observed in cells treated with 2'-FL, in both experiments. 2'-FL was determined to be non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.

C.3.2.2 Studies Conducted with Other 2'-FL Preparations

A summary of the genotoxicity studies conducted on the chemically synthesised 2'-FL produced by Glycom and on the ingredient produced with fermentation by Jennewein is presented in the table below. The results are consistent with the product-specific studies summarised above and confirm that 2'-FL is not expected to be genotoxic.

Table C.3.2.2-1 Summary of Genotoxicity Studies Conducted on 2'-FL

Test	Concentration	Metabolic Activation	Result	Reference
2'-FL Produced by Fermentation				
Bacterial reverse mutation (*S.* Typhimurium strains and *Escherichia coli* strain	Concentrations of up to 5,000 µg/plate (2'-FL produced by fermentation, Jennewein)	± S9	Negative	Jennewein Biotechnologie GmbH, 2015
In vivo micronucleus assay in Crl:CD (SD) rats	Doses of 500, 1,000, or 2,000 mg/kg body weight (2'-FL produced by fermentation, Jennewein)	Not applicable	Negative	Jennewein Biotechnologie GmbH, 2015
2'-FL Produced by Chemical Synthesis				
Bacterial reverse mutation (*S.* Typhimurium strains and *Escherichia coli* strain)	52, 164, 512, 1,600, or 5,000 µg/plate (2'-FL* produced by chemical synthesis)	± S9	Negative	Coulet *et al.* (2014)
L5178Y $tk^{+/-}$ mouse lymphoma assay	Up to 5,000 µg/mL (2'-FL* produced by chemical synthesis)	± S9	Negative	Coulet *et al.* (2014)
Micronucleus assay (cultured peripheral human lymphocytes)	512, 1,600, or 2,000 µg/mL (2'-FL* produced by chemical synthesis)	± S9	Negative	Verbaan (2015b)

* Manufactured by the Applicant; 2'-FL = 2'-*O*-fucosyllactose; S9 = activation with Aroclor 1254-induced rat liver S9.

C.3.3 Repeated-Dose Toxicity Studies

C.3.3.1 Studies Conducted with Glycom's 2'-FL Obtained by Fermentation

The oral toxicity of 2'-FL produced by fermentation, as described herein, was investigated in an adapted subchronic (90-day) oral toxicity study in 7-day-old Wistar [Crl:WI(Han)] rats (Penard, 2015; Appendix VIII). The study was conducted in accordance with the OECD Test Guideline No. 408 (OECD, 1998a,b), with an adaptation to include the use of neonatal rats. The studies were conducted in rats starting on Postnatal Day (PND) 7 to adequately cover the developmental window during which infants will be exposed. Fructo-oligosaccharide (FOS; tradename Orafti® P95), was used as the reference control on the basis that it is currently authorised as an ingredient in infant and follow-on formulae. Doses of the test article were based on the previous results from the 90-day oral toxicity study conducted with chemically synthesised 2'-FL, in which doses of 6,000 mg/kg body weight/day resulted in the unscheduled mortality of 2 pups which was deemed possibly related to treatment (although there was no evidence of a direct toxic effect). The no-observed-adverse-effect level (NOAEL) of the previous study with the chemically synthesised 2'-FL ingredient was reported to be 5,000 mg/kg body weight/day. Test doses of 2,000, 4,000, and 5,000 mg 2'-FL/kg body weight/day were selected for the testing of the 2'-FL ingredient produced by fermentation. The reference, FOS, was tested at a dose of 5,000 mg/kg body weight/day. Rats (10/sex/group) were orally administered by gavage at doses of 0 (water vehicle control), 2,000 (low-dose), 4,000 (mid-dose), or 5,000 (high-dose) mg/kg body weight/day of 2'-FL (97.6% 2'-FL by assay) or the reference compound, FOS, at 5,000 mg/kg body weight/day for 90 or 91 days. Additional groups of 5 males and 5 females were given the control, 2'-FL, or FOS doses for 90 days and were terminated after a 28-day recovery period. Individual dams with reconstituted litters of at least 5 male and 5 female pups were housed in plastic cages until weaning on PND 21. All pups in each reconstituted litter were treated at the same dose level (starting on PND 7). On PND 21, pups were weaned and placed in plastic cages according to sex and dose group such that a total of 5 pups of the same sex and dose group were housed per cage. A standard diet (A04C-10) and water were provided *ad libitum*. Animals were observed twice daily for mortality and morbidity, and clinical observations were performed daily. A detailed clinical examination was performed weekly.

Body weights were assessed at time of randomisation, prior to dosing, twice weekly during the first 8 weeks of the administration period, and then once weekly thereafter. Food intake also was measured twice weekly after weaning and for the first 6 weeks post-weaning, and then once weekly thereafter. Ophthalmological examinations were performed on all animals from the control, high-dose 2'-FL, and FOS groups during the last week of administration. Fasting blood and urine samples were collected from all animals of all groups for clinical pathology analysis (*i.e.*, haematology, coagulation, clinical chemistry, and urinalysis) at the end of the administration period. Clinical pathology also was performed for all animals from all groups at the end of the recovery period. Complete necropsy was performed and selected organs were removed and weighed for all animals at the end of the treatment period or at the end of the 4-week recovery period. Histopathological examinations of all organs and tissues were performed for all animals in the control, high-dose 2'-FL, and FOS groups at the end of the administration period. Kidneys from all females in the low- and mid-dose groups and in all recovery groups also were microscopically examined.

No test article-related mortalities occurred during the study[5]. The majority of animals receiving the reference compound presented with liquid faeces, which was also observed in mid- and high-dose animals receiving 2'-FL. Mid- and high-dose animals receiving 2'-FL also had soiled urogenital regions. Hypersalivation, abnormal foraging and/or pedalling were observed in animals receiving the reference compound and also in the mid- and high-dose groups receiving 2'-FL from Day 35 onward, with the incidence of these clinical signs being most prominent in the high-dose 2'-FL group. No test article-related ophthalmological findings were observed. No remarkable effects in body weight, body weight gain, or food consumption were observed. No toxicologically relevant effects in tibia length, reflex and physical development, time to sexual maturation, learning capacity, memory, motor activity (as evaluated in the Morris water maze), exploratory behaviour, or general movement (as evaluated in the open-field test) were observed at any dose level.

Minor differences in certain haematological parameters were deemed to be of no toxicological significance[6]. Triglyceride concentrations were decreased in mid- and high-dose males receiving 2'-FL compared with the water control group and the FOS reference group. Cholesterol concentrations also were decreased in low-, mid-, and high-dose males receiving 2'-FL and in females receiving mid- and high-dose 2'-FL as compared to the water control group. Individual urea concentrations also were noted to be high for a few animals receiving high-dose 2'-FL. However, it was noted that overall, these changes in serum chemistry parameters were low in magnitude and/or within the normal historical control data range; furthermore, these differences in serum parameters were not observed following the recovery period. Thus, it was concluded that no adverse effect of treatment was observed in serum biochemical parameters.

No test article-related differences in urinalysis parameters were observed between treatment groups and the water control or reference compound[7]. No treatment-related differences in organ weights or macroscopic observations were observed between rats receiving 2'-FL and the control and reference groups. No evidence of treatment-related effects in histological observations was observed in animals receiving 2'-FL compared to control and reference groups.

A NOAEL of 5,000 mg/kg body weight/day, the highest dose tested, was determined for this study.

[5] One death of a mid-dose male and one death of a female animal receiving the reference compound FOS were deemed to be accidental and related to test article administration error, respectively.

[6] A minor decrease in mean red blood cell count was observed in females receiving the reference compound and 2'-FL and a slightly prolonged mean prothrombin time was observed in animals receiving the reference compound or mid- and high-dose 2'-FL. However, these were of low magnitude or within the normal historical control data range and thus were considered to be not toxicologically relevant.

[7] Minor differences in mean specific gravity or mean urinary pH were not considered of toxicological significance as they were within the normal historical control data range and of low magnitude.

C.3.3.2 Studies Conducted with Other 2'-FL Preparations

A) 90-Day Oral Toxicity Study Conducted with Chemically Synthesised 2'-FL

The oral toxicity of 2'-FL has been evaluated in another 90-day study in rats conducted in accordance with the OECD Test Guideline No. 408 (OECD, 1998a,b), with an adaptation to include the use of neonatal rats (Coulet *et al*., 2014). The 2'-FL used in the study (Glycom AS, Denmark) was produced by chemical synthesis and had a purity of 99% (by HPLC, on a dry weight basis). Neonatal (PND 7) Wistar [Crl:WI(Han)] pups[8] were administered 2'-FL by oral gavage at doses of 0 (water vehicle control), 2,000 (low-dose), 5,000 (mid-dose), or 6,000 (high-dose) mg/kg body weight/day from PND 7 to up to 13 weeks of age. A reference control group (15 rats/sex/group) was administered 6,000 mg/kg body weight/day of oligofructose (OF). 2'-FL was well tolerated at doses of up to 5,000 mg/kg body weight/day for 13 weeks, with the only notable observations reported by the authors being transient lower body weight gain[9] and coloured/liquid faeces during the first few days of the administration period. The authors reported three unexplained deaths, 1 male and 1 female in the 6,000 mg/kg group on Day 2, and 1 male in the FOS control group on Day 12. No cause of death could be determined following gross or histopathological investigations, and were not associated with marked changes in any other safety indices measured at the end of the study. Nevertheless, due to the unexplained deaths, a NOAEL of 5,000 mg/kg body weight, the mid-dose, was reported by the study authors.

This study (Coulet *et al*., 2014) has been reviewed within a recent novel food opinion published by EFSA (2015a) for the use of 2'-FL in infant formula and conventional food products. That agency stated that "*based on the decrease in the relative kidney weight in the 2'-FL high-dosed female group, two unexplained deaths in the high-dose 2'-FL group and high-dosed female group, and the significant changes in the haematological and clinical blood parameters in the 2'-FL mid- and high-dosed group, the Panel considers that the no observed adverse effect level (NOAEL) is 2 000 mg/kg body weight per day*." (EFSA, 2015a). The haematological effects were limited to slight reductions in (<5%) red blood cell count that were not consistent between sexes and were not associated histopathological or gross pathological correlates. Changes in clinical chemistry parameters were limited to dose responsive reductions in AST levels[10] in both sexes. AST levels were similarly decreased by a comparable magnitude in both males and females of the FOS group (*i.e*., positive control). In the absence of further clinical chemistry, haematological or histopathological correlates, the reduction in AST levels were not considered an adverse finding by the GRAS Expert Panel (GRN 546) (U.S. FDA, 2015a).

B) 90-Day Feeding Study Conducted with 2'-FL Obtained by Fermentation (Jennewein)

A 90-day feeding study has been conducted with the 2'-FL material produced by Jennewein. The study was conducted in accordance with GLP and with consideration of OECD Test Guideline No. 408 (OECD, 1998a,b). The 2'-FL used in the study was produced by microbial fermentation and had a purity of 94.1% (see Table 5.4-1). A summary of the study results, as well as full study report, are publicly available for review in GRN 571 (Jennewein Biotechnologie GmbH, 2015; U.S. FDA, 2015b). In brief, the study was conducted using groups of 10 four-week-old male and female CD® Crl:CD Sprague-Dawley rats randomised to 1 of 2 treatment allocations administered 2'-FL in the diet at concentrations of 0 or 10% for 90 days. Additional groups of 3 and 9 animals per sex were included in the control (0%) and treatment (10%) groups, respectively, and used exclusively for blood sampling. 2'-FL was well tolerated by the test animals, with the only notable effects being sporadic pale coloration of the faeces in the treatment group, which was attributed to the presence of undigested 2'-FL. No animal deaths were reported. No test article-related effects in body weight, body weight gain, food consumption, water consumption, neurological parameters, haematological and blood biochemical parameters, urinalysis,

[8] The control and high-dose groups each consisted of 15 males and 15 females, while the low- and mid-dose groups each consisted of 10 males and 10 females.

[9] No significant difference in food consumption was observed between the groups receiving 2'-FL and the control group.

[10] AST levels decreased by up to ~20% (P<0.05) in the male and female high dose 2-FL groups.

ophthalmological observation, organ weights, or macroscopic or histopathological findings were observed. The NOAEL was concluded by Jennewein to be 10% dietary concentration of 2'-FL (the only dose tested in this study), which corresponds on average to 7,660 mg/kg body weight/day in males and 8,720 mg/kg body weight/day in females.

C.3.4 Neonatal Piglet Feeding Study

The tolerability of 2'-FL produced by fermentation was also evaluated in a neonatal piglet model. The study was conducted in compliance with the OECD and FDA's principles of GLP (Hanlon and Thorsrud, 2014). Domestic Yorkshire Crossbred farm piglets received liquid diets [11] containing 0 (control), 200 (low-dose), 500 (mid-dose), or 2,000 (high-dose) mg/L of 2'-FL, corresponding to doses of 0, 29.37, 72.22, or 291.74 mg/kg body weight/day in males and 0, 29.30, 74.31, and 298.99 mg/kg body weight/day in females, respectively. The 2'-FL was produced by fermentation as described within GRN 571 and was characterised as the following: 97.9% 2'-FL, 4.2% water, 0.37% ash, 3.3% difucosyllactose, 1.9% fucosyl-galactose, and <50 ppm protein (Jennewein, 2015; U.S. FDA, 2015b) [12]. Piglets were administered the liquid diet for the first 3 weeks of life (modelling the first 3 weeks of human life) [13]. Due to the imbalance of the number of male and female piglets available at the initiation of the study, animals were assigned to treatment groups strategically to ensure the control and highest dose group had an equal distribution of male and female animals. Thus, 6, 8, 7, and 6 male piglets, and 6, 4, 5, and 6 female piglets were assigned to the control, low-, mid-, and high-dose groups, respectively. Diets were offered orally *via* a feeding bowl filled by hand size times per day at a dose volume of 500 mL/kg body weight/day for at least 20 consecutive days during the study. Individual piglet doses were based on the most recent body weight measurements and food consumption was measured and recorded daily during the study. All animals were monitored for morbidity, mortality, injury, availability of food, body weight, and food efficiency during the study period. Blood samples were obtained on Study Days 7 and 21 and evaluated for haematological (including leukocytes, erythrocytes, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, platelet count, reticulocytes, neutrophils, lymphocytes, monocytes, eosinophils, basophils, and other cells), coagulation (including activated partial thromboplastin time, prothrombin time, and fibrinogen), and blood biochemical parameters (including sodium, potassium, chloride, calcium, phosphorus, alkaline phosphatase, bilirubin, gamma glutamyltransferase, aspartate aminotransferase, alanine aminotransferase, sorbitol dehydrogenase, urea nitrogen, creatine, total protein, albumin, triglycerides, cholesterol, and glucose. Urine samples were collected at terminal necropsy and evaluated for urine volume, specific gravity, and pH. Organ weights were recorded for the brain, heart, kidney, large intestine, liver, small intestine, and spleen and the pH of the intestinal contents of the cecum and colon also were recorded. Histopathological examinations were performed on the organs listed above, as well as the eye, gall bladder, stomach, gross lesions, lung with bronchi, mesenteric lymph nodes, pancreas, and Peyer's patch.

Dietary administration of 2'-FL was noted by the study investigators to be "well tolerated". All animals survived to the scheduled necropsy. Clinical observations included the following: watery faeces were noted in 2 low-dose males and 2 low-dose females, 1 mid-dose male and 2 mid-dose females, and 3 high-dose males and 2 high-dose female. One high-dose male and 2 high-dose females exhibited a lack of appetite on 1 day. Lastly, 1 low-dose female exhibited a lack of appetite for 2 days during the study. These observations in appetite were not considered to be toxicologically relevant as there was no dose relationship and there were no differences in body weight observed between treated piglets and controls. No differences in food consumption were observed between treatment groups. No test-

[11] Land O'Lakes® ProNurse® Specialty Milk Replacer from Purina Animal Nutrition, Gray Summit, Missouri.

[12] No detectable levels of lactose, glucose, galactose, fucose, or protein were noted. The purity of the test article (based on mass balance) was established to be 103.87%.

[13] It is noted that neonatal piglets have similarities to human infants in respect to presence of specific digestive enzymes, nutrient absorption, gut closure, dietary requirements, microbial population, and gut transit time. This makes neonatal piglets a good animal model for the first 3 months of life for human infants.

article related effects in haematological, coagulation, or blood biochemical parameters were noted[14]. Similarly, no test article-related effects on urinalysis parameters were noted. No gross or histopathological findings were associated with the test article[15]. Although there was a statistically significant increase in the absolute weights of the heart and kidneys for low-dose males, there was not a difference in the relative (to body weight) organ weights and thus were not considered to be test article-related.

It was thus concluded that the addition of 2'-FL to milk replacer at concentrations of up to 2,000 mg/L was well tolerated by neonatal farm piglets and did not result in adverse health effects or impact piglet growth at doses equivalent to 291.74 mg/kg body weight/day in males and 298.99 mg/kg body weight/day in females.

C.4 Toxicological Data for LNnT

C.4.1 Test Articles Used in the Toxicological Assessments

Glycom's LNnT ingredient obtained by fermentation has been the subject of comprehensive toxicological assessments, including a 90-day oral toxicity study, an *in vitro* bacterial reverse mutation assay, and an *in vitro* micronucleus assay. In addition, toxicological testing has also been conducted with chemically synthesised LNnT (also manufactured by Glycom), as well as an LNnT preparation obtained from a fermentation process using yeast (Prieto, 2005). The toxicological dataset pertaining to LNnT is summarised in the following sections; a comparison of the characteristics of the test articles used in these studies is provided in the table below for reference.

Table C.4.1-1 Comparison of LNnT Test Articles Used in Toxicological Studies

Parameter	LNnT Preparation		
	LNnT Produced by Fermentation, as Described Herein	**Glycom A/S Coulet *et al.* (2013)**	**Prieto (2005)**
Test Article Purity	97.9%	98.9%	Not reported
Manufacture Process	Fermentation	Chemical synthesis	Coupled yeast fermentation and enzymatic conversion
Production Organism	*Escherichia coli* K-12	NA	Yeast (*Candida famata*; ATCC 32550) *and* *Escherichia coli* JM101 for overexpression of enzymes
Specification			Specifications not reported. Authors reported that the LNnT was "practically devoid of other carbohydrates and organic contaminants with the exception of lactose" (present at less than 2%). The test material also was free of heavy metals, microbial toxins, and microbial contamination.
Assay by HPLC	≥95.0%	≥95.0%	
Water	≤9.0%	≤9.0%	
Ash, sulfated	≤0.4%	≤0.4%	
Aerobic mesophilic total plate count	≤500 CFU/g	≤500 CFU/g	

[14] A statistically significant increase (125%) in alanine aminotransferase concentration was observed in high-dose males; however, there were no significant differences in the other clinical chemistry markers for toxicity and no differences in absolute and relative liver weight were observed compared to controls. Furthermore, there were no histopathological correlates and thus this observation was deemed to be not test article-related.

[15] Animals in all treatment groups and the controls had variable minimal-to-mild focal acute inflammation within the keratinized portion of the nonglandular stomach. There was no clear dose dependence and in the absence of similar findings in the stomach, this observation was not definitively linked to the test article. It was further noted by the study investigators that the incidence matched historical control data from the same facility.

Table C.4.1-1 Comparison of LNnT Test Articles Used in Toxicological Studies

Parameter	LNnT Preparation		
	LNnT Produced by Fermentation, as Described Herein	Glycom A/S Coulet *et al.* (2013)	Prieto (2005)
Preclinical Studies Conducted	Ames test *In vitro* micronucleus assay 90-day oral toxicity study	Bacterial reverse mutation test Mouse lymphoma cell gene mutation test *In vitro* micronucleus assay 90-day oral toxicity study	Bacterial reverse mutation test and other *in vitro* mutagenicity studies (not further specified) 28-day oral toxicity studies

CFU = colony-forming units; HPLC = high-performance liquid chromatography; NA = not applicable

C.4.2 Mutagenicity/Genotoxicity

C.4.2.1 Studies Conducted with LNnT Obtained by Fermentation

The mutagenicity of LNnT (94.4% LNnT by assay), as described herein, was evaluated in a bacterial reverse mutation assay in *S. typhimurium* strains TA98, TA100, TA1535, and TA1537 and in *E. coli* strain WP2uvrA in the presence or absence of metabolic activation (S9), using the plate incorporation and pre-incubation methods (Verspeek-Rip, 2016). The study was conducted in accordance with the Organization for Economic Cooperation and Development (OECD) principles of Good Laboratory Practice (GLP) and according to OECD Test Guideline No. 471 (OECD, 1997a, 1998a). The water vehicle served as a negative control for all strains. One of the following compounds was employed as a positive control for different strains in assays conducted in the absence of S9: 2-nitrofluorene (TA98, TA1537, pre-incubation assay), methylmethanesulfonate (TA100), sodium azide (TA1535), ICR-191 (TA1537, direct plate assay), 9-aminoacridine (TA1537), or 4-nitroquinoline n-oxide (WP2uvrA). For assays conducted in the presence of S9, 2-aminoathracene was employed as the positive control.

Using the plate incorporation method, bacterial strains were treated with LNnT at concentrations of 52, 164, 512, 1,600, or 5,000 µg/plate in the presence or absence of S9. For the pre-incubation method, bacterial strains also were incubated with LNnT at concentrations of 52, 164, 512, 1,600, or 5,000 µg/plate in the presence or absence of S9. No cytotoxicity or precipitation was observed in any strain treated with LNnT in any of the experiments. Treatment with LNnT did not result in a biologically significant increase in the number of revertant colonies compared with the negative control at any concentration in both experiments either in the presence or absence of S9. In contrast, positive control agents substantially induced an increase in the number of revertant colonies compared to the negative control. Thus, LNnT was determined to be non-mutagenic under the conditions of the bacterial reverse mutation assay in the presence or absence of exogenous metabolic activation at concentrations up to 5,000 µg/plate.

The genotoxicity of LNnT produced by fermentation (94.4% LNnT by assay) was further investigated in an *in vitro* micronucleus assay conducted in cultured peripheral human lymphocytes (Verbaan, 2016). This study also was conducted in compliance with the OECD principles of GLP and according to OECD Test Guideline No. 487 (OECD, 1998a, 2014). Mitomycin C and colchicine were used as the positive controls in the absence of metabolic activation (S9 mix) and cyclophosphamide was used as the positive control in the presence of S9 mix. Water was used as the negative control. In the short-term exposure experiment, lymphocytes were incubated with LNnT at concentrations of 512, 1,600, or 2,000 µg/mL for 3 hours in the presence or absence of S9, following which the cells were rinsed and incubated for another 24 hours prior to scoring. In the long-term exposure experiment, cells were treated with LNnT at concentrations of 512, 1,600, or 2,000 µg/mL for 24 hours in the absence of S9. At least 1,000

binucleated cells and 1,000 mononucleated were scored for micronuclei under each treatment condition.

In both experiments, there were no signs of precipitation or cytotoxicity (as determined by the cytokinesis block proliferation index) observed in cells treated with LNnT at any concentration. No statistically or biologically significant increases in the frequency of mono- or bi-nucleated cells with micronuclei were observed in cells treated with LNnT, in both experiments. The positive controls produced the expected responses. Thus, LNnT was determined to be non-clastogenic and non-aneugenic in human lymphocytes under the conditions of the assay.

C.4.2.2 Studies Conducted with Other LNnT Preparations

Prieto (2005) reported that LNnT (produced by a coupled yeast /*E. coli* JM101 system) was not mutagenic in the Ames test or in other *in vitro* mutagenicity studies; however, details on these studies were not provided.

The potential mutagenicity of Glycom's chemically synthesised LNnT (purity of 98.9%) has been evaluated in the bacterial reverse mutation assay (Ames test) using *S. typhimurium* strains TA98, TA100, TA1535, TA1537, and TA102 in the presence or absence of metabolic activation (S9), using the plate-incorporation and pre-incubation methods (Coulet *et al*., 2013). This study was conducted in compliance with the OECD principles of GLP and according to OECD Testing Guideline No. 471 (OECD, 1997a, 1998a). The water vehicle served as a negative control for all strains. One of the following compounds was employed as a positive control, depending on the strain, in assays conducted in the absence of S9: 2-nitrofluorene (TA98), sodium azide (TA100 and TA1535), 9-aminoacridine (TA1537), or t-butyl hydroperoxide (TA102). For assays conducted in the presence of S9, 2-aminoathracene was employed as the positive control. In the first experiment using the plate incorporation method, bacterial strains were treated with LNnT at concentrations of 52, 164, 512, 1,600, or 5,000 µg/plate. In the second experiment using the pre-incubation method, bacterial strains were incubated with LNnT at concentrations of 492, 878, 1,568, 2,800, or 5,000 µg/plate. No cytotoxicity or precipitation was observed for any of the strains treated with LNnT in the presence or absence of S9. In both experiments, treatment with LNnT did not result in significant increases in the number of revertants compared with the negative control at any concentration either in the presence of absence of S9. In contrast, positive control agents substantially induced the number of revertant colonies compared to the negative control. Thus, LNnT was determined to be non-mutagenic in the Ames test at concentrations up to 5,000 µg/plate.

The mutagenic potential of LNnT (purity of 98.9%) was further investigated in an *in vitro* mammalian cell gene mutation test in L5178Y tk+/- mouse lymphoma cells (Coulet *et al*., 2013). This study was conducted in compliance with the OECD principles of GLP and according to OECD Testing Guideline No. 476 (OECD, 1997b, 1998a). Methylmethanesulfonate (MMS) and cyclophosphamide were used as the positive controls in the absence and presence of S9, respectively, while water was used as the negative control. In the first experiment, cells were incubated with LNnT at concentrations ranging from 1.4 to 4,250 µg/mL for 24 hours without S9. In the second experiment, cells were treated with LNnT at concentrations ranging from 418 to 4,250 µg/mL for 4 hours with or without S9. In both experiments, no precipitation or cytotoxicity was observed in cells treated with LNnT at any concentration. Also in both experiments, no statistically or biologically significant increases in the frequency of mutations were observed in cells treated with LNnT, with or without S9. Therefore, LNnT was determined to be non-mutagenic in L5178Y tk +/- mouse lymphoma cells.

Lastly, LNnT was evaluated in an *in vitro* micronucleus assay in cultured peripheral human lymphocytes (Verbaan, 2015c). In the study, conducted in accordance with OECD principles of GLP and in accordance to OECD Testing Guideline No. 487 (OECD, 2014), cultured peripheral human lymphocytes were incubated with LNnT (purity of 98.9%) at a concentration of 512, 1,600, or 2,000 µg/mL for either

3 hours (in the presence and absence of S9) or for 24 hours (in the absence of S9) and the number of mono- and binucleated cells were recorded. LNnT did not induce a statistically significant or biologically relevant increase in the number of mono- and binucleated cells with micronuclei in the presence or absence of S9. The positive controls, mitomycin C cyclophosphamide, and colchicine produced the expected responses. Therefore, it was concluded by the study investigators that LNnT was not clastogenic or aneugenic under the conditions of the study.

C.4.3 Repeat Dose Toxicity Studies

6.4.3.1 Studies Conducted with Glycom's LNnT Obtained by Fermentation

The oral toxicity of LNnT, as described herein, was investigated in an adapted subchronic (90-day) oral toxicity study in 7-day-old Wistar [Crl:WI(Han)] rats (Penard, 2016). The study was conducted in accordance with the OECD Principles of Good Laboratory Practices (GLP) (OECD, 1998a), OECD Test Guideline No. 408 (OECD, 1998b) with an adaptation to include the use of neonatal rats. The studies were conducted in neonatal rats starting on PND 7 to adequately cover the developmental window during which infants will be exposed. Fructo-oligosaccharide (FOS; tradename Orafti® P95), was used as the reference control on the basis that it is currently authorised as an ingredient in infant and follow-on formulae. Doses of the test article were based on the previous results from the 28-day oral toxicity study conducted with synthetic LNnT, in which a NOAEL of 5,000 was established. Therefore, test doses of 1,000, 2,500, and 5,000 mg LNnT/kg body weight/day were selected for the testing of the LNnT ingredient produced by fermentation. The reference, FOS, was tested at a dose of 5,000 mg/kg body weight/day. Rats (10/sex/group) were orally administered by gavage at doses of 0 (water vehicle control), 1,000 (low-dose), 2,500 (mid-dose), or 5,000 (high-dose) mg/kg body weight/day of LNnT (94.4% LNnT by assay) or the reference compound, FOS, at 5,000 mg/kg body weight/day for 90 or 91 days. Additional groups of 5 males and 5 females were given the control, LNnT, or FOS doses for 90 days and were terminated after a 28-day recovery period. Individual dams with reconstituted litters of at least 5 male and 5 female pups were housed in plastic cages until weaning on PND 21. All pups in each reconstituted litter were treated at the same dose level (starting on PND 7). On PND 21, pups were weaned and placed in plastic cages according to sex and dose group such that a total of 5 pups of the same sex and dose group were housed per cage. A standard diet (A04C-10) and water were provided *ad libitum*. Animals were observed twice daily for mortality and morbidity, and clinical observations were performed daily. A detailed clinical examination was performed weekly. Body weights were assessed at time of randomisation, prior to dosing, twice weekly during the first 8 weeks of the administration period, and then once weekly thereafter. Food intake also was measured twice weekly after weaning and for the first 6 weeks post-weaning, and then once weekly thereafter. Ophthalmological examinations were performed on all animals from the control, high-dose LNnT, and FOS groups during the last week of administration (Day 90). Fasting blood and urine samples were collected from all animals of all groups for clinical pathology analysis (*i.e.*, haematology, coagulation, clinical chemistry, and urinalysis) at the end of the administration period. Physical development endpoints included pinna unfolding (evaluated daily from PND 2 until confirmation), eye opening (evaluated daily from PND 12 until confirmation), incisor eruption (evaluated daily from PND 7 until confirmation), and left tibia length (evaluated weekly from PND 7 onward) for all pups. For females, detection of day of vaginal opening and body weight on the day of the occurrence was evaluated daily from PND 28 until detection. In males, detection of the day of balano preputial skinfold cleavage and body weight on the day of the occurrence was evaluated daily from PND 38 until detection. Reflex tests were conducted on all pups and assessments of neurological development were evaluated by water maze (on Weeks 8 or 9) and open field test (on Week 10). Complete necropsy was performed and selected organs were removed and weighed for all animals at the end of the treatment period or at the end of the 4-week recovery period. Histopathological examinations of select organs and tissues were performed for all early decedents and animals administered the vehicle control or high-dose LNnT.

No test article-related mortalities occurred during the study. Isolated occurrences of hypersalivation were noted in 1 high-dose male and 3 high-dose females receiving LNnT but were considered by the study authors to be unrelated to LNnT administration. No test article-related ophthalmological findings were observed. No remarkable effects in body weight, body weight gain, or food consumption were observed. No toxicologically relevant effects in tibia length, reflex and physical development, time to sexual maturation, learning capacity, memory, motor activity (as evaluated in the Morris water maze), exploratory behaviour, or general movement (as evaluated in the open-field test) were observed at any dose level.

Statistically significant differences in haematology parameters were noted [16], however lacked a dose-response relationship, were minimal in magnitude (*i.e.*, ~10% or less), and were considered unrelated to LNnT administration. Similarly, no test article-related effects in serum clinical chemistry parameters were observed [17] and any statistically significant differences in serum clinical chemistry parameters were considered to be unrelated to LNnT administration.

A statistically significant increase in urine volume in high-dose animals and a statistically significant decrease in specific gravity were noted in high-dose animals compared with controls. However, these were deemed by the study investigators to be incidental and of no toxicological relevance due to the lack of dose-response or histopathological changes in the kidney. No treatment-related differences in organ weights, macroscopic observations, or histological observations were observed between rats receiving LNnT and the control and reference groups.

A NOAEL of 5,000 mg/kg body weight/day, the highest dose tested, was determined based on the results of this study.

C.4.3.2 Studies Conducted with Other LNnT Preparations

A) LNnT Produced by Chemical Synthesis

Glycom's chemically synthesised LNnT ingredient was investigated in an adapted subchronic (90-day) oral toxicity study using 7-day-old Han Wistar rats. The toxicological studies package also includes a 14-day dose-range finding study and a 28-day repeated-dose study (Coulet *et al.*, 2013). Juvenile rats were selected for these studies to supplement the standard OECD 408 protocol to better reflect the intended use and target population (*i.e.,* infants) (OECD, 1998b; Barrow, 2007). Thus, the studies were conducted in rats starting at PND 7 to adequately cover the developmental window during which infants will be exposed. In all studies, OF (also known as short-chain fructo-oligosaccharide) was used as the reference control on the basis that it is currently used in infant formula. The OF used has been determined to be GRAS for use in infant formula with no questions from FDA (GRN 392) (U.S. FDA, 2012). The doses selected for these studies (*i.e.,* 1,000, 2,500, and 5,000 mg/kg body weight/day) and overall experimental design were based on a published subchronic 90-day toxicity study conducted in rats investigating the safety of other oligosaccharides (*i.e.*, galacto-oligosaccharides and fructo-oligosaccharides) for use in infant formulas (Anthony *et al.*, 2006). It should be noted that the highest dose tested of 5,000 mg/kg body weight/day was the highest dose able to be tested as administration of macronutrients at higher levels may result in nutritional imbalances, which would complicate a proper safety assessment. The highest dose tested also corresponds to 5 times the current limit dose of 1,000 mg/kg body weight/day recommended in OECD Testing Guideline No. 408 (OECD, 1998b).

[16] These changes included a decrease activated partial thromboplastin time in high-dose males and females (≤10.1%), decreases in fibrinogen in low-, mid-, and high-dose males and high-dose females (≤12.6%), decrease in red blood cell count in low-dose females (≤6.0%), and decreases in absolute (but not relative) reticulocytes in females receiving LNnT and FOS (≤10.3%).
[17] These changes included decreases in mean total bilirubin concentration in low-, mid-, and high-dose males and low-dose females, decreases in calcium in low-dose males, decrease in glucose in low-dose females, and an increase in creatinine in high-dose females.

In the dose-range finding study for subsequent longer term toxicity studies, Wistar [Crl:WI(Han)] pups (5/sex/group) were administered by gavage 0 (water vehicle control), 1,000, 2,500, or 5,000 mg/kg body weight/day of Glycom's LNnT (purity of 98.9%) from PND 7 to weaning on PND 20 (Coulet *et al*., 2013). A reference control group was orally administered 5,000 mg OF/kg body weight/day for the same period of time. Individual dams with litters of at least 5 male and 5 female pups were housed in plastic cages. One pup of each sex in each litter was randomly allocated to 1 of the 5 groups. A standard diet (A04C-10) and water were provided *ad libitum*. Animals were observed twice daily for mortality and morbidity, and clinical observations were performed daily. Detailed clinical examination was performed weekly, and body weights were measured on PND 1, 4, 7, 10, 14, 17, and 20. Feed intake was not measured. All animals were euthanised at the end of the administration period on PND 21 and macroscopic examinations were performed. No mortality and no abnormalities in clinical signs, body weights, and necropsy were observed in rats administered LNnT throughout the administration period. In rats administered OF, erythema in the anal region was observed from Days 5 to 8 of the administration period, which was associated with diarrhoea. A transient reduction in body weight gain during the first 3 days of the administration period also, was observed. Based on these findings, the authors determined that the highest dose of LNnT considered suitable for longer-term rat studies was 5,000 mg/kg body weight/day, the highest dose tested.

In the subsequent 28-day repeated-dose study, juvenile Wistar [Crl:WI(Han)] rats were administered Glycom's LNnT (purity of 98.9%) *via* gavage at doses of 0 (water vehicle control), 1,000 (low-dose), 2,500 (mid-dose), or 5,000 (high-dose) mg/kg body weight/day for 28 consecutive days beginning on PND 7 (Coulet *et al*., 2013). The study was conducted in compliance with the OECD Principles of Good Laboratory Practices (GLP) and according to OECD Testing Guideline No. 407 (OECD, 1998a, 2008). A reference control group received 5,000 mg/kg body weight/day of OF for the same period of time. Individual dams with litters of at least 5 male and 5 female pups were housed in plastic cages until weaning on PND 21. One pup of each sex in each litter was randomly allocated to one of the 5 groups. On PND 21, pups were weaned and placed in plastic cages according to sex and dose group such that a total of 5 pups of the same sex and dose group were housed per cage. A standard diet (A04C-10) and water were provided *ad libitum*. The low- and mid-dose groups consisted of 10 male and 10 female rats, while the control, high-dose, and OF groups consisted of 15 male and 15 female rats. Animals were observed twice daily for mortality and morbidity, and clinical signs were recorded daily. A detailed clinical examination was performed weekly. Body weights were assessed at time of randomisation, prior to dosing, then every 2 days until weaning on PND 21 and twice weekly thereafter during the administration and recovery periods. Food and water intake also were measured twice weekly after weaning on PND 21 during the administration and recovery periods. Ophthalmology was performed on all animals in the control, high-dose LNnT, and OF groups during the last week of administration. Fasting blood and urine samples were collected from all animals for clinical pathology evaluation (*i.e.,* haematology, serum chemistry, and urinalysis) at the end of the administration and recovery periods. Blood samples were drawn from the retro-orbital sinus under isoflurane anaesthesia. Twenty (20) animals from each group (10/sex) were euthanised and necropsied on the day after the last day of dosing. The remaining animals in the vehicle control, high-dose, and OF groups (5/sex/group) were monitored over a 14-day recovery period and then euthanised and necropsied. Complete necropsy was performed and selected organs were removed and weighed from all animals. Histopathological examinations of all organs and tissues were performed for all animals in the control, high-dose, and OF groups.

Four males (2 control and 2 low-dose) died after blood sampling on the day of necropsy likely due to the high volume of blood (3.5 mL) taken. In addition, 1 female in the OF group died on Day 4; the cause of death was not determined. The majority of animals in the OF group exhibited soft, liquid, yellow-coloured faeces and erythema on the urogenital area from Day 1 of the study until weaning (Day 13 to 14 of the study). Similar findings were noted in the high-dose LNnT group prior to weaning, but this was reported to occur at a much lower frequency and in only a few animals. These findings were not observed in the control or low- and mid-dose LNnT groups. The authors noted that gastrointestinal

effects, such as increased soft stool, diarrhoea, and intestinal weights, as well as caecal distension, have been reported in other preclinical studies on OF, and that these findings have been attributed to the non-digestible, fermentable nature of OF as soluble fibres. There were no other compound-related clinical observations reported. In addition, there were no compound-related ophthalmological abnormalities observed in any group.

No significant differences in mean body weight or adverse effects on mean body weight gain were reported between the LNnT groups and the control group. Lower mean body weight gain was reported in the OF group during the first 3 days of the administration period, which resulted in a significantly lower mean body weight between Days 2 and 10 compared to the control group. There were no significant differences in body weight gain between the OF and control groups thereafter, and mean body weights were similar among all groups from Day 12 onwards to the end of the administration period. No statistically significant changes in food or water intake were observed during the administration or recovery period.

Haematology revealed statistically significant increases (30%) in total white blood cell (WBC) and absolute monocyte counts in the high-dose female LNnT group compared to the control group. These changes were considered by the investigators to be slight in nature, and therefore, incidental changes of no toxicological significance. Moreover, changes in WBC and absolute monocyte counts were not observed in females in the 90-day study detailed below. No changes in relative monocyte counts were observed. Slight, yet statistically significant, differences (<10%) were noted in males of the mid- and high-dose groups, consisting of the following: decreased reticulocytes and mean cell volume (MCV) and increased mean corpuscular haemoglobin concentration. However, since these changes were slight in magnitude and not associated with any clinical or histopathological changes, these findings were considered to have no biological or toxicological significance. Furthermore, the haematological variations observed in rats administered LNnT were not observed in the 90-day study (apart from the decrease in reticulocytes). No significant differences in coagulation parameters were observed.

Several slight, yet statistically significant, differences (<5%) in clinical chemistry parameters were observed between the LNnT or OF groups and the control group, including lower mean serum total protein (all dose groups), albumin (high-dose group), and globulin concentrations (all dose groups) in LNnT-administered females and in both males and females of the OF group. The authors noted that these differences tended to be slightly more pronounced in the OF group, but that there were no significant differences in the albumin/globulin ratio between the control group and the LNnT or OF groups. Moreover, the changes observed in females of the LNnT groups were non-dose-dependent as changes in these groups were of similar magnitude. Furthermore, variations observed in both males and females administered LNnT were not observed in the 90-day study. Several other statistically significant differences in clinical chemistry parameters observed between the control and LNnT groups consisted of decreased sodium concentrations in high-dose males and females (<1%) and increased glucose concentrations in all male dose groups (12 to 15%); however, these findings were not considered to be toxicologically relevant due to their small magnitude of change. Statistically significant decreases in alanine aminotransferase (ALT) activity (13%) and aspartate aminotransferase (AST) activity (19%) were observed in high-dose females and a statistically significant decrease (11 to 17%) in AST activity was observed in all male dose groups. While the decreased ALT levels observed in male LNnT groups appeared dose-dependent, changes in other liver enzymes [AST and aspartate aminotransferase (ALP)] were not observed in males and no association with histopathological findings were observed. Furthermore, a reduction in liver enzyme activity levels would not be indicative of liver toxicity. None of the other changes observed were dose-dependent. Similar changes observed in the LNnT groups were noted in the OF group when compared to the control group. No compound-related effects on urinalysis parameters were observed.

No compound-related changes in organ weights or macroscopic findings were observed. Histopathological examination revealed lobular degeneration/atrophy of acinar cells from the pancreas

in one male of the high-dose LNnT group. This finding is consistent with the normal background of spontaneous lesions observed in Wistar rats, and therefore, was not considered to be compound-related. A minimal increase in zymogen content of acinar cells was observed focally in 2 high-dose females (statistical analysis not performed). Given the focal distribution and the low incidence and severity of this finding, the authors considered this observation to be incidental and related to individual variation. All other microscopic findings noted also were considered by the authors to be incidental and unrelated to LNnT administration. Based on the results of this study, the NOAEL for LNnT determined for this study is 5,000 mg/kg body weight/day, the highest dose tested, in juvenile Wistar [Crl:WI(Han)] rats.

In the subchronic 90-day toxicity study, Wistar [Crl:WI(Han)] pups (15/sex/group) were orally administered by gavage 0 (water vehicle control), 1,000 (low-dose), 2,500 (mid-dose), or 5,000 (high-dose) mg/kg body weight/day of LNnT (purity of 98.9%) from PND 7 to up to 13 weeks of age (Coulet *et al*., 2013). A 28-day recovery period was included in the study. The study was conducted in compliance with the OECD principles of GLP and in accordance with the OECD Testing Guideline No. 408 (OECD, 1998a,b). A reference control group (15 rats/sex/group) administered 5,000 mg/kg body weight/day of OF were included in the study. Individual dams with litters of at least 5 male and 5 female pups were housed in plastic cages until weaning on PND 21. One pup of each sex in each litter was randomly allocated to one of the 5 groups. On PND 21, pups were weaned and placed in plastic cages according to sex and dose group such that a total of 5 pups of the same sex and dose group were housed per cage. A standard diet (A04C-10) and water were provided *ad libitum*. Animals were observed twice daily for mortality and morbidity, and clinical observations were performed daily. A detailed clinical examination was performed weekly. Body weights were assessed at time of randomisation, prior to dosing, and then twice weekly during the administration and recovery periods. Food intake also was measured twice weekly after weaning on PND 21 during the administration and recovery periods. Ophthalmology was performed on all animals from the control, high-dose LNnT, and OF groups during the last week of administration. Fasting blood and urine samples were collected from all animals of the control, high-dose LNnT, and OF groups for clinical pathology analysis (*i.e.*, haematology, clinical chemistry, and urinalysis) at the end of the administration period. Blood samples were drawn from the retro-orbital sinus under isoflurane anaesthesia. Clinical pathology also was performed for all animals from all groups at the end of the recovery period. Twenty (20) animals from each group (10/sex) were euthanised and necropsied on the day after the last day of dosing. The remaining animals in all groups (5/sex/group) were monitored over a 4-week recovery period to evaluate the regression of any observed effects and then euthanised and necropsied. Complete necropsy was performed and selected organs were removed and weighed for all animals. Histopathological examinations of all organs and tissues were performed for all animals in the control, high-dose LNnT, and OF groups, as well as for all animals in all recovery groups. The spleen and pancreas from all animals in the low- and mid-dose groups also were microscopically examined.

One male and 1 female in the mid-dose group and 1 female in the OF group died after dosing on either Day 13 or 14. Although there were no evident signs noted at necropsy, the authors considered that the most likely cause of death in these animals was intubation error or trauma during administration of the test compounds. As in the 28-day study, yellow liquid faeces and reddening of the urogenital region were observed in the majority of the animals in the OF group up to the time of weaning. Similar findings were occasionally observed in the high-dose LNnT group before weaning, but again this was reported to occur at a much lower frequency than in the OF group and in only a few animals. These findings were not observed in the control or low- and mid-dose LNnT groups. No other compound-related clinical signs were observed, and no compound-related ophthalmological findings were noted.

No statistically significant differences in mean body weights were observed between the LNnT and control groups. A marginal decrease in body weight gain was observed in the high-dose LNnT group compared to the control group during the first 3 days of administration; however, the effect was not statistically significant. No significant differences in body weight gain were observed in the low- or mid-

dose groups compared to the control group at any time point. As observed in the 28-day study, animals administered OF also displayed a transient, but statistically significant, reduction in body weight gain over the first 3 days of administration when compared to controls, resulting in a significant decrease in mean body weight. Animals in the OF groups subsequently displayed increases in growth rate such that mean body weights were comparable to controls by weaning. No notable variations in body weight gain were observed from weaning to the end of the administration period, and there were no compound-related effects on post-weaning food intake during either the administration or recovery period.

Several statistically significant differences in haematology parameters were observed in the high-dose LNnT group at the end of the administration period compared to the control group. These included decreased (≤6%) mean haemoglobin (Hb) concentration and packed cell volume (PCV) in females and decreased reticulocyte (17%) and platelet counts (10%) in males; the effects observed in males also were observed in OF-administered males. WBC counts were also significantly reduced by 14% in high-dose LNnT males, which was accompanied by a significant 10% reduction in the absolute lymphocyte count. At the end of the 4-week recovery period, WBC counts remained significantly lower in males administered LNnT at all doses compared to control males. Similar findings of reduced WBC were noted in males administered OF compared to controls. The authors noted that the haematological changes observed in females were generally within the range of historical control data and that all changes noted were not sufficient in magnitude to be considered as adverse. Furthermore, none of the statistically significant findings were associated with changes in clinical parameters or histopathological abnormalities. The authors thus considered that the haematological changes observed were of no biological or toxicological significance. Several statistically significant differences in clinical chemistry parameters were observed in the high-dose LNnT group compared to the control group. These changes included the following: slightly decreased (1%) sodium concentrations in males and females, decreased urea (13%) and cholesterol (18%) concentrations and increased glucose concentration (13%) in males, decreased AST activity (23%) in males, and decreased ALT activity (12%) in females. Similar changes in sodium, glucose, AST, and ALT levels were also observed in the 28-day study. However, all findings were considered by the authors to be incidental with no association with histopathological findings, and therefore, are non-adverse in nature. Moreover, the reduction in liver enzyme activity levels observed would not be indicative of liver toxicity. No compound-related effects on urinalysis parameters were observed.

No compound-related adverse effects on organ weights were observed. In addition, no compound-related macroscopic or microscopic lesions were observed at necropsy following the administration or recovery periods. All findings were considered to be incidental and due to inter-individual variations. Based on the results of this 90-day study, the authors determined the NOAEL for LNnT to be 5,000 mg/kg body weight/day, the highest dose tested, in Wistar [Crl:WI(Han)] rats. The authors also noted that, in terms of gastrointestinal symptoms, LNnT was better tolerated than OF during the first 2 weeks of administration (pre-weaning period) and was without adverse organ effects in all studies as assessed macroscopically at necropsy and through histopathological examinations. Therefore, the authors concluded that these findings further support the safety of LNnT for use in infant foods.

B) LNnT from Coupled Fermentation with Yeast and *E. coli*

Prieto (2005) also investigated the potential toxicity of LNnT in 4-week studies. The LNnT test article investigated in these studies was produced using glycosyltransferase derived from yeast. In the 28-day study, 12 litters (5/sex/litter) of Cri:CD®BR rat pups (15 days old) were administered 0 (control), 10, 200, or 400 mg/kg body weight/day of LNnT (purity not reported) *via* gavage. Parameters evaluated included urinalysis, haematology, faecal analysis, and gross pathology. There were no significant differences in any of the parameters measured reported among groups. In the dietary study, 31- to 37-day-old rats (sex, strain, and number not reported) were fed diets containing 1 or 5% of LNnT for a period of

4 weeks[18]. The dietary levels of LNnT provided are equivalent to a dose of approximately 1,000 and 5,000 mg/kg body weight/day, respectively, based on an average body weight of 100 g reported for young rats (U.S. FDA, 1993). It is unknown whether a control group was included in this study. Detailed clinical chemistry and histopathological examinations were conducted (no further details were provided). No test article-related adverse effects or macroscopic and microscopic changes were observed following LNnT administration. No further details on these studies were provided in the publication by Prieto (2005) although the publication provides a general indication that LNnT lacks toxicity in rats at the doses administered.

C.5 Clinical Data

C.5.1 Infant Study Conducted with 2'-FL in Combination with LNnT

The safety of Glycom's 2'-FL and LNnT has been evaluated in a randomised, double-blinded, controlled, multi-centre, parallel-design study conducted in healthy, full-term infants. In this study, infants were provided a standard term infant formula supplemented with 2'-FL (providing 1.0 to 1.2 g 2'-FL/L of reconstituted formula)[19] in combination with LNnT (providing 0.5 to 0.6 g LNnT/L reconstituted formula)[20] from 0 to 6 months of age (Puccio *et al.*, 2017). The full study report is provided in Appendix IX. Both 2'-FL and LNnT were supplied by Glycom. Infants were aged 0 to 14 days at enrolment and clinic visits were scheduled at 1, 2, 3, 4, 6, and 12 months of age. A comparator group receiving a standard whey-predominant starter infant formula without HiMOs was included as controls. The infants were exclusively fed the test or control formulas for the first 4 months of age. Complementary foods were allowed to be introduced beginning at 4 months of age. At 6 months of age, the infants in both study groups (test and control formula) were switched to an intact protein, cow's milk–based, follow-up formula without HiMOs for feedings through to 12 months of age. Body weight gain through to 4 months was evaluated as the primary endpoint, with secondary endpoints being additional anthropometric measures including body weight, body length, and head circumference, as well as gastrointestinal tolerance, behavioural patterns, and morbidity through to age 12 months. Additionally, the effects of 2'-FL and LNnT supplementation on the intestinal microbiota profile was also assessed in this study (Alliet *et al.,* 2016; Steenhout *et al*., 2016). The outcomes of this analysis are described further in Section E.

A total of 175 infants were enrolled in the study. The mean weight gain in the test group was similar and "non-inferior" to the mean weight gain in the control group in both the intent-to-treat population and the per protocol population. Based on the recommendations from the American Academy of Pediatrics, weight gain in the test group receiving the formula supplemented with LNnT and 2'-FL was defined as "non-inferior" to controls if the lower bound of the one-sided 97.5% confidence interval on the difference between the test and control groups was greater than the non-inferiority margin of -3 g/day. Mean daily formula consumption was similar between groups at all time points examined.

The proportion of infants who experienced at least one serious adverse event was 6.8% (6 infants) in the group receiving the test formula containing 2'-FL and LNnT and 11.5% (10 infants) in the standard infant formula group. The proportion of infants experiencing at least one adverse event in the first 4 months of the study was similar between the test and control groups. One infant receiving the test formula experienced an adverse event considered to be related to the study formula (the infant developed a cow's milk protein allergy). Twelve infants in the test group and 11 infants in the control group

[18] There is some ambiguity with respect to the duration of this study that was reported by Prieto (2005). In the *Materials and Methods* section of this publication, it was indicated that these rats were administered diets containing LNnT for 4 weeks. However, within the *Results* section, the dietary experiment was referred to as being 4 months in duration.
[19] Analytical results indicated a concentration range of 1.04 to 1.14 g/L (SD 0.073 to 0.08 g/L) of 2'-FL in test formula (unpublished data).
[20] Analytical results indicated a concentration range of 0.52 to 0.61 g/L (SD 0.028 to 0.033 g/L) of LNnT in test formula (unpublished data).

experienced adverse events resulting in the discontinuation of the study formula. Overall, the incidence of adverse events was not significantly different in infants fed the test formula compared to the control standard formula.

The mean length, head circumference, and body mass index of the infants were not statistically significantly different between the test and control groups at any study visit. Furthermore, growth data indicated the formula containing 2'-FL and LNnT supported age-appropriate normal infant growth when compared to the World Health Organization (WHO) Growth Standards. Digestive symptoms (infant flatulence, spitting up, vomiting) and behaviour patterns (restlessness/irritability, colic) were comparable between the 2 groups, with the exception of softer stool and fewer night-time wake-ups in infants receiving 2'-FL and LNnT compared to controls. Infants receiving the HiMOs also had significantly fewer parental reports of bronchitis, lower respiratory tract infections, antipyretic use, and antibiotic use compared to controls and endpoints examined. The study authors concluded that "Infant formula with 2'-FL and LNnT is safe, well tolerated, and supports age-appropriate growth."

C.5.2 Other Studies Conducted in Infants

A) 2'-FL in Combination with Galacto-oligosaccharides (GOS) in Infant Formula

The safety of 2'-FL (Inalco SpA, Milan, Italy) was evaluated in a published randomised, controlled, double-blinded, prospective study conducted in healthy, full-term, singleton infants (Marriage *et al.,* 2015). Infants were enrolled within Day of Life (DOL) 5 and were provided 1 of 3 formulae: i) a standard, milk-based, commercially available infant formula containing 2.4 g GOS/L (control formula); ii) the standard formula supplemented with 0.2 g 2'-FL/L and 2.2 g GOS/L; or iii) the standard infant formula supplemented with 1.0 g 2'-FL/L and 1.4 g GOS/L. All formula had a caloric density of 64.3 kcal/dL (comparable to human milk) and contained a total of 2.4 g/L of non-digestible oligosaccharides [21]. The mothers of infants receiving formulae were instructed to feed the study formulae as their infant's sole source of nutrition until DOL 119. A comparator (reference) group comprised of infants consuming human milk (by breast and/or bottle) also was included [22]. The primary endpoint was body weight gain from DOL 14 to DOL 119. Secondary endpoints included measures of tolerance and other anthropometric parameters. The presence of 2'-FL also was evaluated in blood and urine collected from a subset of infants at DOL 42 and 119 and in the comparator group at DOL 42. Moreover, the effects of 2'-FL supplementation on biomarkers of immune function among infants in this study have been separately reported in a publication by Goehring *et al.* (2016). The outcome of this analysis are described in Section E.

A total of 338 infants completed the study; the number of infants discontinuing the study formulae was not different among the formula-fed groups. No significant differences in body weight gain, body weight during clinical visits, length, or head circumference were observed between the formulae groups and the human milk reference group. The mean daily volume of study formula consumed during the study period was similar between the control and test formula groups; however, between enrolment and DOL 28, the control group consumed significantly more formula than the group receiving the formula supplemented with 0.2 g 2'-FL (661 mL/day compared to 614 mL/day, p = 0.024). The mean consumption values for other time points and for the other formula group were not disclosed by the authors. All formulae were well tolerated, and no significant differences in the overall percentage of infants with adverse events or serious adverse events were observed between infants receiving the experimental formulae and the standard formula. Average stool consistency, number of stools per day, and the percent of feedings associated with spit-up or vomit were comparable between all groups.

[21] The standard milk-based infant formula contained 2.4 g GOS/L. The 2 experimental formulae contained 0.2 g 2'-FL + 2.2 g GOS per liter; and 1.0 g 2'-FL + 1.4 g GOS/L, respectively.

[22] Infants belonging to the breastfed reference group were not to receive greater than 240 mL of infant formula per week.

2'-FL was detected in the plasma and urine of infants provided 2'-FL in formula and in infants consuming human milk. The plasma concentrations of 2'-FL on DOL 42 reflected the amount of 2'-FL present in the feeds (*i.e.,* human milk > formula containing 1.0 g 2'-FL/L > formula containing 0.2 g 2'-F/L); however, the mean plasma concentrations were not similarly correlated for samples obtained on DOL 119 [23]. No 2'-FL was detected in the plasma of infants fed the un-supplemented standard milk-based commercial formula containing GOS. Mean urine concentrations of 2'-FL were greatest in infants fed human milk and the formula containing 1.0 g 2'-FL/L, followed by infants fed the formula containing 0.2 g 2'-FL/L and the un-supplemented standard formula. The relative excretion was calculated to be 1.44 and 1.12% for the group receiving the 0.2 g 2'-FL/L and 1.0 g 2'-FL/L formulae, respectively.

Based on the results of the study, the investigators concluded that the feeding of infant formula with a caloric density similar to that of human milk resulted in comparable growth rates to that of human milk-fed infants and that formulae supplemented with 2'-FL were well tolerated.

B) 2'-FL in Combination with Short-Chain Fructo-Oligosaccharides in Infant Formula

The gastrointestinal tolerance of an infant formula containing 2'-FL in combination with short-chain fructo-oligosaccharides (scFOS) was evaluated in a prospective, randomised, multi-centre, double-blinded controlled study in full-term, singleton infants (Kajzer *et al.*, 2016). A total of 121 infants were enrolled between 0 to 8 days of age and were either breastfed (reference group) or assigned to receive 1 of 2 experimental milk-based formulas containing a caloric density of 643 kcal/L. The control formula did not contain oligosaccharides (composition not further detailed), and the test formula was supplemented with 0.2 g/L 2'-FL combined with 2.0 g/L scFOS. A record of milk/formula intake, stool patterns (including average mean rank stool consistency), anthropometrics, were obtained until 35 days of age and parental questionnaires (not further detailed) were also collected.

No significant differences in mean rank stool consistency were observed between groups. No significant differences among stool consistency were reported between groups, although the average number of stools per day was significantly higher in the breastfed group compared to both formula groups. No significant differences in formula intake, number of study formula feedings per day, anthropometric data, or percent feedings with spit up/vomit were observed by the investigators. The study authors concluded that the test formula was well tolerated, with anthropometric data and feeding characteristics similar to infants fed control formula or breastmilk.

C) Studies Conducted with LNnT from Coupled Fermentation with Yeast / E. coli

One study on the consumption of LNnT produced using a coupled yeast / *E. coli* fermentation process in human infants was identified in the literature (Prieto, 2005). In this study, consisted of a double-blind, randomised, placebo-controlled study in which 228 healthy infants and young children (6 to 24 months old) were provided with a 220 mg/L of LNnT (22 kcal/30 mL) formula or control formula *ad libitum* for a period of 112 days (16 weeks). The study was conducted in accordance with the Independent Ethics Committee and Institutional Review Board, and the Declaration of Helsinki. The test subjects were monitored for 16 weeks, during which oropharyngeal swabs were carried out for *Streptococcus pneumoniae* every 2 weeks to determine the effects of LNnT on oropharyngeal colonisation with *S. pneumoniae*. Average consumption of both formulas was slightly greater than 500 mL/day per subject, and the variability of formula ingestion was not statistically significant between the control and the LNnT group. LNnT was well tolerated by subjects. A slightly higher body weight and length was observed in the LNnT group; however, these effects were not considered statistically significant. Although colonisation rate of *S. pneumoniae* was higher in the LNnT group when compared to the control group throughout the study, this finding was not statistically significant. During the biweekly

[23] This finding was hypothesized by the study investigators to be due to developmental changes in the structure and function of the gastrointestinal tract mucosa and compositional transformation of the intestinal microbiota leading to a less permeable gut and better utilization of 2'-FL by microbiota populations, respectively.

health assessment, ear status was examined by monitoring for otitis media (middle ear infection) or other signs of inflammation or disruption of the ear canal. Based on the results of the ear examination, the authors classified subjects as having "normal ears", "abnormal ears", or "indeterminable" (assigned when the child was unwilling or unavailable to undergo the ear examination). The LNnT group displayed a statistically significant lower rate of overall "abnormal ears" when compared to the control group. However, the significance was not maintained when antibiotic administration was considered a covariate for the analysis. Thus, based on the results of this study, LNnT was well tolerated in infants and was without adverse effects on growth and ear health at a concentration of 220 mg/L, which is consistent with the lower range of LNnT levels present in mature human breast milk (Kunz *et al.*, 2000; Erney *et al.*, 2001).

Prieto (2005) also reported that, previous to the infant study, 2 studies on adults were carried out in which adults were administered sequential bolus dosages of up to 10 g a day of LNnT without adverse effects reported. No further study details were provided in the publication.

C.5.3 Studies Conducted in Adults

The safety and tolerability of LNnT produced by Glycom was investigated in a randomised, placebo-controlled, double-blind, parallel-design study in which healthy adult volunteers (51 men and 49 women; mean age of 36.0 years) were provided either LNnT or 2'-FL alone, or in combination at different doses for 2 weeks (Elison *et al.*, 2016). A comparator control group receiving glucose as a placebo was also included. The intervention groups used in the study are summarised in the table below. All interventions were provided as daily bolus doses. Test articles were provided in powder form and participants were instructed to dissolve the powder in approximately 250 mL of water prior to intake in the morning with breakfast. Compliance was evaluated using a subject diary in which subjects were instructed to record the intake of the test article, which was confirmed by the collection of empty and un-opened bottles at the end of the intervention period.

Table C.5.3-1 Interventions Used in the Two-Week Healthy Adult Study (Elison *et al.*, 2016)

Group No.	Daily Dose of LNnT (grams)	Daily Dose of 2'-FL (grams)
1	0	20
2	0	10
3	0	5
4	20	0
5	10	0
6	5	0
7	6.67	13.33
8	3.33	6.67
9	1.67	3.33
Control	2 grams Dextropure (glucose)	

2'-FL = 2'-*O*-fucosyllactose; LNnT = lacto-*N*-neotetraose

Adverse events were monitored during the study. Blood samples were collected at baseline and at the end of the intervention period (2 weeks) and evaluated for standard haematological and blood biochemistry parameters. Faeces were collected at baseline and at the end of the intervention period (2 weeks) and evaluated for calprotectin, secretory IgA, glucose, galactose, lactose, and short-chain fatty acids). Gastrointestinal symptoms were evaluated using the Gastrointestinal Symptom Rating Scale (GSRS) and changes in bowel habits were assessed using the Bristol Stool Form Scale (BSFS). Faecal DNA was also extracted to assess microbiota composition (by 16S rRNA sequencing) at baseline, during the first week of supplementation, and at Week 2. The effects of 2'-FL and LNnT on microbiota composition are described in Section E.

All adverse events reported during the study were judged to be "mild" and there were no cases of premature discontinuation from the trial due to adverse events. Most adverse events were judged to be "possibly" related to the test article; however, many symptoms were noted by the study investigators to be common and difficult to ascertain whether they were related to the test article, to normal day-to-day variation, or to increased awareness of gastrointestinal symptoms during the trial period.

Haematological and blood biochemistry analyses obtained at the 2-week time point remained within the normal range for all subjects and any minor changes over the course of the study compared to baseline values were not considered clinically relevant. The GSRS scores indicated that LNnT and 2'-FL were well tolerated. When compared to the placebo control, individuals receiving the highest dose of LNnT (20 g) reported an increased bloating and passing of gas, and harder stools and individuals receiving 10 g LNnT reported increased passing of gas after the 2-week intervention period. However, scores generally remained at a level of "mild discomfort" or less. The BSFS scores indicated a mild tendency to softer stools in individuals provided the high dose of LNnT or 2'-FL over the course of the study compared to baseline, but differences were small and clinically irrelevant.

Overall, the results support that the consumption of LNnT and 2'-FL, either alone or in combination, at the doses tested, was safe and well tolerated in healthy adult men and women. Acute intake of a bolus dose of 20 g of LNnT (or 2'-FL) may represent a gastrointestinal tolerability threshold for some individuals; however, bolus exposures of 20 g of LNnT are highly unlikely to be experienced by the consumer given the proposed use-levels and consequently required food intakes that would lead to such intakes.

C.6 Safety of the Production Microorganism

C.6.1 History of Consumption

Glycom's 2'-FL and LNnT are obtained by the fermentation of *E. coli* K-12 (DH1) SCR6 and *E. coli* K-12 (DH1) MP572, respectively. As described in Section B.4.3, *E. coli* K-12 and its derivatives are known as bacterial "safety-strains". Their safety has been well recognised and they are widely used as a host organism in the construction of biotechnologically engineered microorganisms used for the production of food ingredients and food enzymes.

Multiple genetic modifications have been made to *E. coli* K-12 (DH1) SCR6 and *E. coli* K-12 (DH1) MP572, in order to introduce the metabolic pathways needed to allow for the biosynthesis of 2'-FL and LNnT, respectively. A full listing of the genetic modifications made to the production strains is presented in Appendix V-b. The newly expressed proteins are "carbohydrate-active enzymes" ("CAZy"), a panel of enzymes that can degrade, modify, or create glycosidic bonds, and accordingly are involved in the metabolism of complex carbohydrates[24]. For 2'-FL production, the modifications also include changes that allow the strain to grow on sucrose as the sole carbon source. The genetic sequences of the newly introduced proteins are based on those present in various microbial donor strains (see Appendix V-b). The newly introduced proteins do not have an extensive history of consumption *per se*, considering the donor organisms are not widely consumed food sources, and that the resulting proteins are not the same as the carbohydrate-modifying enzymes that are widely used as processing aids by the food industry. Even so, the newly introduced proteins do not pose any safety concerns. The genetic modifications made to the production strains result in the expression of proteins that are involved in the normal carbohydrate processing within their donor microbial (bacterial) sources. When expressed together in the recipient strains, these proteins work in concert to convert the starting carbohydrates (lactose and sucrose for 2'-FL, and lactose, glucose and glycerol for LNnT) into oligosaccharides that are identical to those in human breastmilk. In contrast, bacterial protein toxins (exotoxins) are known to

[24] A database of CAZy enzymes is available at: http://www.cazy.org/Welcome-to-the-Carbohydrate-Active.html.

mediate their pathogenic effects by disrupting cellular processes through various mechanisms such as proteolysis (*e.g.*, tetanus and botulinum), ADP-ribosylation (*e.g.*, cholera, pertussis, and diphtheria), or membrane disruptions through pore formation (Finlay and Falkow, 1997; Popoff, 2018; Wilson *et al.*, 2002). Indeed, bioinformatic searches conducted using the amino acid sequences of the proteins introduced to the *E. coli* K-12 (DH1) SCR6 and *E. coli* K-12 (DH1) MP572 production strains by genetic modification confirmed that there is no relevant homology to known protein toxins or to known allergens (see Section C.6.2).

Additionally, it is important to highlight that during the manufacturing process of 2'-FL and LNnT, these HiMOs are secreted by the production organism. No cellular disruption is required, and the intact production organism is entirely removed by a series of purification steps, as outlined in Section B.4. Absence of the production organism from the final HiMOs ingredients is verified by a number of microbiological purity criteria, including the absence of *E. coli* and *Enterobacteriaceae,* and the absence of residual proteins, residual DNA, and residual bacterial endotoxins (see Section B.5.1). Accordingly, the production strains employed in the manufacture of 2'-FL and LNnT, which have been genetically modified to express the proteins required for the biosynthesis of these HiMOs, do not pose any safety concerns. This is further supported by the absence of any adverse effects in an extensive dataset of toxicological studies that have been conducted specifically with Glycom's 2'-FL and LNnT obtained by fermentation (see Sections C.3 and C.4).

C.6.2 Bioinformatic Searches for Homology with Known Toxins and Allergens

The Basic Local Alignment Search Tool (BLAST) maintained by the National Center for Biotechnology Information (NCBI) was used to search for homology between the sequences of the introduced proteins in the production strains [*E. coli* K-12 (DH1) SCR6 and MP572] against those of known animal venom proteins/toxins and virulence factors in databases maintained by UniProt (UniProtKB/Swiss-Prot Tox-Prot and UniProtKB/Swiss-Prot/TrEMBL). Details of this assessment are presented in Appendix X-a. The results of these bioinformatic searches suggest that the proteins introduced into the productions trains do not share homology or structural similarity with any known animal venom proteins/toxins or virulence factors. Additionally, the allergenic potential of the introduced proteins in the production strains was evaluated against the sequences of known allergens in the Allergen Online database and the NCBI Entrez database. As detailed in Appendix X-b, the results of these bioinformatic searches suggest that the newly introduced proteins in the production strains do not pose any allergenicity concerns.

It is important to reiterate that the production organism is completely removed in the intact form, and a series of purification steps are subsequently applied during the production of Glycom's 2'-FL and LNnT. Accordingly, no proteins are detected in the final 2'-FL and LNnT ingredients. All batches of 2'-FL and LNnT are specified to contain <0.01% protein as analysed using a modified Bradford method, and analyses of production batches have demonstrated the level of protein to be below the quantification limit of 0.0017% (see Section B.6).

C.7 Assessments by Authoritative Bodies

C.7.1 European Union

2'-FL and LNnT Obtained by Chemical Synthesis

2'-FL may be chemically synthesised by the use of L-fucose and D-lactose as substrates (with benzyl-2'-FL as an intermediate). Similarly, LNnT may be chemically synthesised by the use of D-lactose as a substrate (with benzyl-LNnT as an intermediate). These methods of manufacture result in products that are identical in structure to the ingredients produced by fermentation (as described herein).

In the EU, a novel food application was submitted for the use of 2'-FL obtained from chemical synthesis in 2014. At the request of the European Commission, the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) delivered a scientific opinion on the safety of 2'-FL as a novel food ingredient (EFSA, 2015a). Concurrently, a novel food application was submitted for LNnT obtained from chemical synthesis, and the NDA Panel also delivered a Scientific Opinion on the safety of LNnT (EFSA, 2015b). Overall, the NDA Panel concluded that the use of 2'-FL and LNnT:

- Is safe for infants (up to one year of age) when added to infant and follow-on formulae, in combination with lacto-*N*-neotetraose (LNnT), at concentrations up to 1.2 g/L of 2'-FL and up to 0.6 g/L of LNnT, at a ratio of 2:1 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 1.2 g/L of 2'-FL (alone or in combination with LNnT, at concentration up to 0.6 g/L, at a ratio of 2:1); and

- Is safe when added to other foods at the proposed uses and use levels.

Formal EU approval for the use of 2'-FL was acquired in 16 March 2016 under *Commission Implementing Decision (EU) 2016/376 of 11 March 2016 authorising the placing on the market of 2'-O-fucosyllactose as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council* (Commission Decision 2016/376) (EU, 2016a). Formal approval of LNnT was acquired in 11 March 2016 under *Commission Implementing Decision (EU) 2016/375 of 11 March 2016 authorising the placing on the market of lacto-N-neotetraose as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council* (EU, 2016b).

The authorised uses and maximum levels for chemically synthesised 2'-FL are laid down under Annex II of Commission Decision 2016/376 and Commission Decision 2016/375 (EU, 2016a,b). These legislations permit the inclusion of 2'-FL and LNnT in combination in infant formulae and follow-on formulae at levels of 1.2 and 0.6 g/L, respectively, in the final product ready for use (marketed as such or reconstituted as instructed by the manufacturer). 2'-FL and LNnT are also permitted for use in a range of other conventional food categories (see Section D.5 of this application).

2'-FL and LNnT Obtained by Fermentation

In 2016, Glycom submitted an application to the Food Safety Authority of Ireland (FSAI) for an opinion on the substantial equivalence of their 2'-FL and LNnT obtained by microbial fermentation to the chemically-synthesised ingredients that are authorised for use under Commission Decision 2016/376 and Commission Decision 2016/375 (EU, 2016a,b). The FSAI issued a favourable opinion for both ingredients produced by fermentation, concluding that:

- "T*he FSAI is satisfied that the information provided by the applicant demonstrates that 2'FL produced by fermentation is substantially equivalent to the chemically synthesised comparator which was authorised for the EU market to Glycom A/S by Commission Implementing Decision (EU) 2016/376 (EU, 2016a). The uses and maximum use levels of the novel 2'FL will be the same as for the EU-authorized synthetic 2'FL.*" (FSAI, 2016a); and

- *"The FSAI is satisfied from the information provided that lacto-N-neotetraose (LNnT) produced by Glycom A/S using microbial fermentation is substantially equivalent to the synthetic counterpart authorised for the EU market by Commission Implementing Decision (EU) 2016/375 in terms of composition, nutritional value, metabolism, intended use and level of undesirable substances.*" (EU, 2016b; FSAI, 2016b)

The intent to market Glycom's 2'-FL and LNnT produced by fermentation have been notified to the European Commission (European Commission, 2016a,b).

In addition, the 2'-FL produced by fermentation from Jennewein Biotechnologie GmbH (Jennewein) is approved for use as a novel food ingredient under *Commission Implementing Decision (EU) 2017/2201 of 27 November 2017 authorising the placing on the market of 2'-fucosyllactose produced with Escherichia coli strain BL21 as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council* (EU, 2017). As outlined in Annex II of this Commission Implementing Decision, 2'-FL is authorised for use in infant formulae and follow-on formulae at levels of up to 1.2 g/L in the final product ready for use (marketed as such or reconstituted as instructed by the manufacturer).

C.7.2 United States

2'-FL and LNnT Produced by Chemical Synthesis

The FDA has issued a "no questions" response to the conclusion that chemically synthesised 2'-FL and LNnT is GRAS for use as an ingredient in term infant formula at a maximum level of 2.4 g 2'-FL per L (GRN 546) and 0.6 g LNnT per L (GRN 547), as consumed (U.S. FDA, 2015a,c). The ingredients are also GRAS for use in various foods [including baked goods and baking mixes, beverages and beverages bases, coffee and tea, dairy product analogues, infant and toddler foods, grain products and pastas, milk (whole and skim), processed fruits and juices, processed vegetables and juices, and sugar substitutes] at maximum levels ranging from 0.084 to 2.4 g of 2'-FL per serving (GRN 546), and 0.02 to 1.2 g LNnT per serving (GRN 547) (U.S. FDA, 2015a,c).

2'-FL and LNnT Produced by Fermentation

Glycom's 2'-FL ingredient obtained from fermentation with an *E. coli* K-12 derived strain has been concluded as GRAS for use in term infant formula at a maximum level of 2.4 g 2'-FL per L, as well as for use in various other foods [including other baby foods and drinks for infants and young children such as toddler formula, beverages and beverages bases, dairy product analogues, grain products and pastas, milk (whole and skim), milk products, processed fruits and juices, and processed vegetables and juices] at maximum levels ranging from 0.084 to 2.4 g of 2'-FL per serving. The GRAS status was notified to the FDA in 2016 (GRN 650) and the FDA has issued a "no questions" response to Glycom's conclusion that 2'-FL was GRAS for its proposed uses (U.S. FDA, 2016a)

Glycom's LNnT ingredient obtained from fermentation with *E. coli* K-12-derived strain has been concluded as GRAS for use in term infant formula at a maximum level of 0.6 g LNnT per L, as well as for use in various other foods [including beverages and beverages bases, dairy product analogues, infant and toddler foods, grain products and pastas, milk (whole and skim), milk products, and processed fruits and juices] at maximum levels ranging from 0.02 to 3.0 g of LNnT per serving. The GRAS status of this LNnT ingredient was notified to the FDA in 2016 (GRN 659) and the FDA issued a "no questions" letter on 29 November 2016 (U.S. FDA, 2016b).

It is further noted that another manufacturer of 2'-FL obtained by microbial fermentation (Jennewein) has also concluded the use of their ingredient to be GRAS when used in term infant formula and toddler formula at levels of up to 2,000 mg 2'-FL per L (GRN 571), to which the FDA has issued a "no questions" response (U.S. FDA, 2015b). This ingredient is prepared from the fermentation of an *E. coli* BL21 (DE3) derived strain.

C.7.3 Israel

2'-FL obtained by fermentation as produced by Jennewein has been authorised as novel food in Israel for use in milk-based infant formulas (ages 0 to 6 months) and follow-on formulas (ages 6 to 12 months), at levels of up to 2.0 g/L of the ready-to-feed product (Israel MOH, 2017).

C.7.4 Singapore

In 2017, the Agri-Food & Veterinary Authority of Singapore (AVA) concluded that 2'-FL obtained by fermentation, as produced by Glycom, is permitted as an ingredient in infant formula (including follow-on formula) for infants 0 to 12 months old and growing up milk (for children aged 12 to 36 months), up to a level of 1.2 grams per litre of reconstituted formula (Singapore Agri-Food & Veterinary Authority, 2017a [personal communication] [25]). Additionally, the AVA concluded that LNnT obtained by fermentation, as produced by Glycom, is permitted for use in the same food categories at levels up to 0.6 grams per litre of reconstituted formula (Singapore Agri-Food & Veterinary Authority, 2017b [personal communication] [26]). The 2'-FL and LNnT ingredients may be used either singly or in combination.

Growing up milks (also termed formulated milks) containing the 2'-FL and LNnT ingredients at the proposed level of use are now permitted for sale in Singapore. With regards to the uses of 2'-FL and LNnT in infant formula and follow-on formula, the infant formula standard within the Food Regulations will need to be amended to allow for this use before the import, manufacture and sale of such products are permitted. This amendment to the Food Regulations is expected to be formally gazetted in mid-2018.

C.8 Summary of Safety Data

The 2'-FL and LNnT ingredients manufactured by Glycom has been accepted for use in the EU and U.S. in the same food categories (*i.e.,* infant formula, follow-on formula, and "toddler milks") and the same (or even higher) use levels as those proposed in Australia/New Zealand. No safety concerns are associated with the use of the source organisms, *E. coli* K-12 (DH1) SRC6 and MP572, that are used to produce 2'-FL and LNnT, respectively. HMOs, including 2'-FL and LNnT, do not undergo any significant digestion in the upper gastrointestinal tract, and are expected to enter the colon largely intact where they are subject to fermentation by the microbiota present.

The safety of 2'-FL and LNnT has been extensively evaluated in a number of preclinical and clinical studies. The pre-clinical toxicological dataset for 2'-FL includes three 90-day oral toxicity studies conducted in rats, a 3-week feeding study in neonatal piglets, and a series of mutagenicity/genotoxicity assays. Of these, a 90-day oral toxicity study, an *in vitro* bacterial reverse mutation assay, and an *in vitro* micronucleus assay were specifically conducted with Glycom's 2'-FL produced by fermentation (*i.e.*, the subject of this application). The NOAEL for 2'-FL produced using fermentation was determined to be 5,000 mg/kg body weight/day, the highest dose tested, based on the results of a 90-day study conducted in Wistar rats starting from PND 7 (Penard, 2015). This NOAEL value is corroborated by the results of another 90-day oral toxicity study conducted with the chemically-synthesised 2'-FL ingredient in which the NOAEL also was determined to be 5,000 mg/kg body weight/day (the mid-dose tested) (Coulet *et al.*, 2014). Further, 2'-FL produced by microbial fermentation has been reported to be well tolerated in a neonatal piglet model when formulated in a milk replacer providing up to 291.74 mg/kg

[25] Singapore Agri-Food & Veterinary Authority (2017a) [personal communication]. *[Response to: Application for the Use of 2'-Fucosyllactose (2'-Fl) in Infant Formula, Follow-On Formula, and Growing-Up Milk Powder]. [Confidential].* Singapore: Agri-Food & Veterinary Authority, Regulatory Administration Group, Regulatory Programmes Department.

[26] Singapore Agri-Food & Veterinary Authority (2017b) [personal communication]. *[Response to: Application for the Use of Lacto-N-Neotetraose (LNnT) in Infant Formula, Follow-On Formula, and Growing-Up Milk Powder]. [Confidential].* Singapore: Agri-Food & Veterinary Authority, Regulatory Administration Group, Regulatory Programmes Department.

body weight/day in males and 298.99 mg/kg body weight/day in females during a 20-day treatment period (Hanlon and Thorsrud, 2014). The results of a bacterial reverse mutation test and an *in vitro* micronucleus assay conducted on the 2'-FL produced by fermentation demonstrate that the ingredient is not mutagenic/genotoxic, which is consistent with the results of studies conducted with other 2'-FL preparations.

Table C.7-1 Summary of the Repeated-Dose Toxicology Studies Conducted with 2'-FL

Species	**Dose** (mg/kg/day bw)	**Duration** (days)	**NOAEL** (mg/kg bw)	Reference
Rat (Wistar)	2,000, 4,000, or 5,000 (2'-FL produced by microbial fermentation, Glycom)	90	5,000	Penard (2015)
Rats (Sprague-Dawley)	0 or 10% 2'-FL in diet (2'-FL produced by microbial fermentation, Jennewein)	90	Approximately 7,660 (males) and 8,720 (females)	Jennewein Biotechnologie GmbH (2015)
Rat (Wistar)	0, 2,000, 5,000 or 6,000 (2'-FL produced by chemical synthesis)	90	5,000	Coulet *et al.* (2014)
Neonatal Piglet	0, 29.37, 72.22, or 291.74 in males 0, 29.30, 74.31, and 298.99 in females (2'-FL produced by microbial fermentation, Jennewein)	21	291.74 in males 298.99 in females	Hanlon and Thorsrud (2014)

2'-FL = 2'-*O*-fucosyllactose; bw = body weight; NOAEL = no-observed-adverse-effect level.

The preclinical toxicological dataset for LNnT includes both dietary and gavage 28-day toxicity studies, two 90-day oral toxicity studies conducted in juvenile rats, 3 *in vitro* bacterial reverse mutation assays, 1 *in vitro* mammalian cell gene mutation test, and 2 *in vitro* micronucleus assays. Of these, a 90-day oral toxicity study, an *in vitro* bacterial reverse mutation assay, and an *in vitro* micronucleus assay were specifically conducted with Glycom's LNnT obtained by fermentation (*i.e.*, the subject of this application). The NOAEL for Glycom's LNnT produced using fermentation was determined to be 5,000 mg/kg body weight/day, the highest dose tested, based on the results of a 90-day study conducted in Wistar rats starting from PND 7 (Penard, 2016). This NOAEL value is corroborated by the results of another 90-day oral toxicity study conducted with the chemically-synthesised LNnT ingredient in which the NOAEL also was determined to be 5,000 mg/kg body weight/day (Coulet *et al.*, 2013). The results of a bacterial reverse mutation test and an *in vitro* micronucleus assay conducted on the LNnT produced by fermentation demonstrate that the ingredient is not mutagenic/genotoxic (Verspeek-Rip, 2016; Verbaan, 2016), which is consistent with the results of studies conducted with other LNnT preparations (Prieto, 2005; Coulet *et al.*, 2013; Verbaan, 2015b).

Table C.7-2 Summary of the Repeated-Dose Toxicology Studies Conducted with LNnT

Species	**Dose** (mg/kg/day bw)	**Duration** (days)	**NOAEL** (mg/kg bw/day)	Reference
Rat (Wistar)	0, 1,000, 2,500, or 5,000 (LNnT produced by microbial fermentation)	90 days	5,000	Penard (2016)
Rat (Wistar)	0, 1,000, 2,500, or 5,000 (LNnT produced by chemical synthesis)	28 days	5,000	Coulet *et al.* (2013)
Rat (Wistar)	0, 1,000, 2,500, or 5,000 (LNnT produced by chemical synthesis)	90 days	5,000	Coulet *et al.* (2013)
Rat (Cri:CD®BR)	0, 10, 200, or 400 (LNnT produced by coupled fermentation with yeast and *Escherichia coli*)	28 days	No effects compared to controls. NOAEL not established by investigators.	Prieto (2005)

Table C.7-2 Summary of the Repeated-Dose Toxicology Studies Conducted with LNnT

Species	**Dose** (mg/kg/day bw)	**Duration** (days)	**NOAEL** (mg/kg bw/day)	Reference
Rat (strain NR)	Equivalent to approx. 1,000 or 5,000 [Dietary concentration of 1 and 5%] (LNnT produced by coupled fermentation with yeast and *Escherichia coli*)	4 weeks[a]	No adverse test article-related effects. NOAEL not established by investigators.	Prieto (2005)

bw = body weight; LNnT = lacto-*N*-neotetraose; NOAEL = no-observed-adverse-effect level.
[a] There is some ambiguity with respect to the duration of this study that was reported by Prieto (2005). In the *Materials and Methods* section of this publication, it was indicated that these rats were administered diets containing LNnT for 4 weeks. However, within the *Results* section, this study was referred to as being 4 months in duration.

Four clinical studies have been conducted in which the safety of 2'-FL (either alone or in combination with LNnT or other non-digestible oligosaccharides) was evaluated; these include 3 studies involving infants and 1 study in adults. Formula supplemented with 1.0 to 1.2 g 2'-FL (in combination with 0.5 to 0.6 g LNnT) for the first 4 months of life was shown to be well tolerated and supported age-appropriate growth (Puccio *et al.*, 2017). This was corroborated by another study published by Marriage *et al.* (2015) in which formula supplemented with 0.2 or 1.0 g 2'-FL/L (in combination with 2.2 and 1.4 g GOS/L, respectively) for the first 4 months of life was well tolerated and did not result in any differences in growth or anthropometric measures, when compared to infants receiving a control infant formula containing GOS only, or to infants in the human breast milk reference group. A formula supplemented with 2'-FL at 0.2 g/L, in combination with 2.0 g/L scFOS, was well tolerated and did not result in any differences in intake, anthropometric data, or average rank stool consistency compared to control formula (without any oligosaccharides) and breastfed groups when administered up to 35 days of age (Kajzer *et al.*, 2016). Additionally, one clinical study was conducted where formula containing LNnT at 0.22 g/L was administered to infants for 4 weeks (Prieto, 2005). No adverse changes in growth were reported, and formula containing LNnT was well tolerated; however, only limited details on this study were provided in the publication (Prieto, 2005). In adults, the results of a safety and tolerability study indicate that consumption of 2'-FL at doses of up to 20 g, LNnT at doses of up to 20 g, or their combination at a 2:1 ratio (for total sum of up to 20 g), when taken as a single bolus dose daily for 2 weeks, was well tolerated and did not result in any deviations in laboratory measures of safety (*i.e.*, haematology and blood biochemistry) compared to normal reference values (Elison *et al.*, 2016).

Table C.7-3 Summary of the Clinical Studies Conducted with 2'-FL and LNnT

Study Population	Duration of Intervention	Study Groups and Test Articles	References
175 healthy full-term singleton **infants** 87-88 per group 0-14 days of age at enrolment	6 months (primary outcome assessment at 4 months)	***Control Formula:*** Intact protein whey based infant formula (670 kcal/L) with LC-PUFA ***Test Formula:*** Same as control, plus **2'-FL** (1.0-1.2 g/L) and **LNnT** (0.5-0.6 g/L) ***Reference group:*** Breast fed infants enrolled at 3 months of age	(Puccio *et al.*, 2017) (Alliet *et al.*, 2016) ***Clinical trial number*** NCT01715246
338 healthy full-term singleton **infants** 101-109 per group day of life 5 at enrolment	4 months (5 to 119 days of age)	***Control Formula:*** Lower Calorie Formula (643 kcal/L) with 2.4 g/L GOS ***Test Formula 1:*** Same as control, plus 2.2 g/L **GOS** and 0.2 g/L **2'-FL** ***Test Formula 2:*** Same as control, plus 1.4 g/L **GOS**, plus 1.0 g/L **2'-FL** ***Reference group:*** Breast fed infants	(Marriage *et al.*, 2015) (Goehring *et al.*, 2016) ***Clinical trial number*** NCT01808105
131 healthy full-term singleton **infants** 42-46 per group 0-8 days of age at enrolment	27 to 35 days (until 35 days of age)	***Control Formula:*** Milk-based infant formula (643 kcal/L), no oligosaccharides ***Test Formula:*** Same as control, plus 2 g/L **scFOS** and 0.2 g/L **2'-FL** ***Reference group:*** Breast fed infants	(Kajzer *et al.*, 2016) ***Clinical trial number*** NCT01808105
228 healthy infants and young children 6 to 24 months old at enrolment	4 weeks	***Control Formula:*** Milk-based formula conforming to nutritional standards in Chile (not further detailed) ***Test Formula:*** Same as control, plus 220 mg/L **LNnT**	Prieto (2005)
100 healthy **adults** 10 per group	14 days	***Control:*** Glucose ***Test Articles:*** • **2'-FL alone:** 5, 10, 20 g/day • **LNnT alone:** 5, 10, 20 g/day • **2'-FL+LNnT *(2:1 ratio)*:** 5, 10, 20 g/day (as sum)	(Elison *et al.*, 2016) ***Clinical trial number*** NCT01927900

2'-FL = 2'-*O*-fucosyllactose; GOS = galacto-oligosaccharides; LC-PUFA = long chain polyunsaturated fatty acids; LNnT = lacto-*N*-neotetraose; scFOS = short-chain fructo-oligosaccharides

Overall, the available data support that the proposed uses of 2'-FL (either alone or in combination with LNnT) in infant formula, follow-on formula, and formulated supplementary foods for young children will be safe and well tolerated. 2'-FL will be added to these formula products at use levels up to 1.2 g/L, either alone or in combination with up to 0.6 g/L of LNnT, which are well within the ranges of these HMOs that have been reported in human breast milk. In addition to its history of safe consumption from breast milk, the safety of 2'-FL and LNnT has been demonstrated through extensive investigations in both preclinical toxicological studies and clinical studies. These include clinical studies that have been conducted to specifically investigate the safety of 2'-FL and LNnT in infant formula consumed by young infants, as well as a tolerability study conducted in adults.

Although clinical studies have only been conducted in young infants and adults, the available dataset also supports that the inclusion of 2'-FL (either alone or in combination with LNnT) in formulated supplementary foods for young children will not result in any adverse effects or negatively impact growth in the target population for such products (*i.e.*, children age 1 to 3 years). The levels of exposure to 2'-FL and LNnT are expected to be the highest among young infants, who represent a particularly vulnerable population given that infant formula serves as the sole source of nutrition. In contrast, older infants and young children consume an increasingly diversified diet, and they also have a larger body weight, meaning that exposure on a mg/kg body weight/basis will be less than those of young infants. Accordingly, intakes modelling conducted as part of the EU novel food application and U.S. GRAS notices for 2'-FL and LNnT demonstrates that infants (up to 12 months of age) have a higher estimated level of intake of 2'-FL and LNnT on both an absolute (g/day) and body weight (mg/kg body weight/day) basis

when compared to young children age 1 to 3 years (see Section D.6). As such, the data obtained from clinical studies in infants are considered to be relevant in supporting the safety of 2'-FL and LNnT for older infants and young children. It is also important to recognize that 2'-FL and LNnT are proposed for addition to formulated supplementary foods for young children (specifically milk products), at levels that are within the ranges of those present in human breast milk, which is considered inherently safe for not only breast-feeding infants, but also young children by extension.

Overall, the totality of data summarised herein demonstrate that the proposed addition of 2'-FL, either alone or in combination with LNnT, to infant formula, follow-on formula, and formulated supplementary foods for young children will be safe and well tolerated.

D. INFORMATION ON DIETARY EXPOSURE TO THE NOVEL FOOD

Information is provided in this Section on the proposed uses and use levels for Glycom's 2'-FL and LNnT ingredient, and accordingly, their anticipated level of exposure. This Section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel Foods), Guideline 3.6.2 (Special Purpose Food – Infant Formula Products), and Guideline 3.6.3 (Special Purpose Foods – Other Foods) of the Food Standards Australia New Zealand Application Handbook (FSANZ, 2016). The corresponding Sections of this Application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	**Required Information Described in the Guideline**	**Section(s) of the Application where this is Addressed**
Guideline 3.5.2 – Novel Foods	D.1 A list of the foods or food groups proposed to or which might contain the novel food ingredient or substance	Section D.1
	D.2 The proposed level of the novel food ingredient or substance for each food or food group	Section D.1
	D.3 For foods or food groups not currently listed in the most recent Australian or New Zealand (NNSs), information on the likely level of consumption	Section D.3
	D.4 The percentage of the food group in which the novel food ingredient is proposed to be used or the percentage of the market likely to use the novel food ingredient	Section D.4
	D.5 For foods where consumption has changed in recent years, information on likely current food consumption	Section D.3
	D.6 Data to show whether the food, or the food in which the novel food ingredient is used, is likely to replace another food from the diet, if applicable	Section D.4
	D.7 Information relating to the use of the novel food or novel food ingredient in other countries, if applicable	Sections D.5, D.6
Guideline 3.6.2 – Special Purpose Food (Infant Formula Products)	B.1 Data to enable the dietary intake or exposure of the target population to be estimated	Section D.1
	B.2 Data on the recommended level of formula consumption for the target population	Section D.6
	B.3 Information relating to the substance (background intake)	Section D.2
Guideline 3.6.3 – Special Purpose Food (Other foods)	B.1 Data to enable the dietary exposure of the target population to be estimated	Section D.1
	B.2 Data on the recommended level of consumption of the special purpose food for the target population	Section D.6

D.1 Proposed Food Uses and Maximum Use Levels

2'-FL is intended for use in infant formula and follow-on formula at a maximum use level of 1.2 g/L of the ready-to-feed or reconstituted formula. 2'-FL may be added on its own, or in combination with up to 0.6 g/L of LNnT. In addition, Glycom also intends to use 2'-FL at up to 1.2 g/L, either alone or in combination with up to 0.6 g/L of LNnT, in formulated supplementary foods for young children, specifically milk products, intended for children age 1 to 3 years. All proposed food uses and maximum use levels are summarised in Table D.1-1.

Table D.1-1 Proposed Food-Uses and Use-Levels for 2'-FL and LNnT in Australia/New Zealand

Category in the FSANZ Code	Proposed Food-Uses	Maximum Proposed Use Level (as Consumed)
Standard 2.9.1 – Infant formula products	Infant formula	1.2 g/L of 2'-FL, alone or with 0.6 g/L of LNnT
	Follow-on formula	1.2 g/L of 2'-FL, alone or with 0.6 g/L of LNnT
	Infant Formula Products for Special Dietary Use	1.2 g/L of 2'-FL, alone or with 0.6 g/L of LNnT
Standard 2.9.3 – Formulated meal replacements and formulated supplementary foods	Division 4 - Formulated supplementary foods for young children	1.2 g/L of 2'-FL, alone or with 0.6 g/L of LNnT

2'-FL = 2'-*O*-fucosyllactose; LNnT = lacto-N-neotetraose.

D.2 Natural Occurrence of 2'-FL and LNnT in the Diet

Typically, infants up to 6 months of age are exclusively fed infant formula (or are otherwise exclusively breastfed) as the sole source of nutrition; as such, consideration does not need to be made with regards to the intake of 2'-FL and LNnT from background dietary sources. With regards to older infants and young children consuming an increasingly diversified diet, the intake of 2'-FL and LNnT from other dietary sources (*e.g.*, milk from other mammals) is considered to be minimal to non-existent. The available data suggest the concentrations of *total milk oligosaccharides* in domestic farm animal milks are much lower than in human milk. For instance, it is estimated that the level of *total milk oligosaccharides* in cow's colostrum is typically 20 times lower than that in human colostrum (Tao *et al.*, 2009). Thus, in the intake assessment for 2'-FL and LNnT from their proposed uses in follow-on formula and formulated supplementary foods for young children, the background intake of 2'-FL and LNnT from the consumption of bovine milk and other mammalian milks are not expected to contribute meaningfully.

D.3 Consumption Information for Foods Not Included in the Most Recent Australian or New Zealand National Nutrition Surveys

Consumption data for infant formula products and formulated supplementary foods for young children that are proposed to contain 2'-FL (either alone or in combination with LNnT) are available in the latest Australia and New Zealand National Nutrition Surveys [*i.e.*, 2011–12 National Nutrition and Physical Activity Survey (NNPAS) component of the 2011–13 Australian Health Survey (2 years and above) (Australian Bureau of Statistics, 2014); the 2008–09 New Zealand Adult Nutrition Survey (15 years and above) (NZ MoH, 2012); and the 2002 New Zealand Children's NNS (5–14 years) (NZ MoH, 2003)]. It is not expected that there have been significant changes in the consumption of these foods since the conduct of these National Nutrition Surveys.

D.4 Percentage of Foods Likely to Contain 2'-FL and LNnT

In deriving the estimated intake of 2'-FL and LNnT from their proposed uses in infant formula and follow-on formula, it can be assumed, as the most conservative measure, that these ingredients will be added to all infant formula products marketed in Australia/New Zealand (including both powdered and

ready-to-feed formulations). For the use of 2'-FL (either alone or in combination with LNnT) in formulated supplementary foods for young children, it is anticipated that these ingredients will be added to all milk products (powdered and ready-to-feed formulations) on the market that are intended for use by young children aged 1 to 3 years. Although in reality, it is unlikely that 2'-FL and LNnT will have 100% market penetration, the application of this assumption in the exposure assessment will provide a conservative estimate of the intake of 2'-FL and LNnT under their proposed condition of use. This assumption was also made in the derivation of the estimated intake of 2'-FL and LNnT from their intended conditions of use in other jurisdictions where these ingredients are already accepted for use (*i.e.*, EU and U.S.).

It is notable that Glycom is seeking exclusive permission to market 2'-FL (either alone or in combination with LNnT) as a novel food. During the initial 15 months of approval, it is expected that one manufacturer will be adding the novel ingredients to one brand on the Australian market, and another brand on the New Zealand market. The current market share, based on volume (kg of product sold), of these representative brands to which 2'-FL (either alone or in combination with LNnT) will be added is presented in Table D.4-1.

Table D.4-1 Market Share for 2'-FL and LNnT During the Exclusivity Period

Product Category	Total Market Size as Volume of Sales ('000)	% Market Share
Representative Brand A (Australia)		
Infant Formula	6206	3.7
Follow-on Formula	4863	3.4
Toddler milk[a]	11146	1.6
Representative Brand B (New Zealand)		
Infant Formula	629	11
Follow-on Formula	782	8
Toddler milk[a]	1279	12

[a] Toddler milks are products that are classified as formulated supplementary foods for young children (Standard 2.9.3, Division 4).

D.5 Approved Uses of 2'-FL and LNnT in Other Countries

D.5.1 European Union

The authorised uses and maximum levels for 2'-FL are stipulated in Annex II of *Commission Implementing Decision (EU) 2016/376 of 11 March 2016 authorising the placing on the market of 2'-O-fucosyllactose as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council (EU, 2016a)*, as replicated in Table D.5.1-1 below.

Table D.5.1-1 Authorised Food Uses and Maximum Use Levels for 2'-FL in the EU

Food Category	Maximum Levels
Uses Authorised Under Commission Implementing Decision 2016/376 (EU, 2016a)	
Unflavoured pasteurised and sterilised (including UHT) milk-based products	1.2 g/L
Unflavoured fermented milk-based products	1.2 g/L beverages 19.2 g/kg products other than beverages
Flavoured fermented milk-based products including heat-treated products	1.2 g/L beverages 19.2 g/kg products other than beverages
Dairy analogues, including beverage whiteners	1.2 g/L beverages 12 g/kg for products other than beverages 400 g/kg for whitener
Cereal bars	12 g/kg
Table-top sweeteners	200 g/kg
Infant formulae as defined in Directive 2006/141/EC (EC, 2006a)and Regulation (EU) No 609/2013	1.2 g/L in combination with 0.6 g/L of lacto-*N*-neotetraose at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
Follow-on formulae as defined in Directive 2006/141/EC (EC, 2006a) and Regulation (EU) No 609/2013	1.2 g/L in combination with 0.6 g/l of lacto-*N*-neotetraose at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
Processed cereal-based food and baby food for infants and young children as defined in Directive 2006/125/EC (EC, 2006b)	12 g/kg for products other than beverages 1.2 g/L for liquid food ready for use, marketed as such or reconstituted as instructed by the manufacturer
Milk-based drinks and similar products intended for young children	1.2 g/L for milk-based drinks and similar products added alone or in combination with lacto-*N*-neotetraose, at concentrations 0.6 g/L, at a ratio of 2:1 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
Dietary foods for special medical purposes as defined in Directive 1999/21/EC (EC, 1999)	In accordance with the particular nutritional requirements of the persons for whom the products are intended
Foods intended for use in energy-restricted diets for weight reduction as defined in Directive 96/8/EC (only for products presented as a replacement for the whole of the daily diet) (EC, 1996)	4.8 g/L for drinks 40 g/kg for bars
Bread and pasta products for people intolerant to gluten as defined in Regulation (EC) No 41/2009 (EC, 2009)[a]	60 g/kg
Flavoured drinks	1.2 g/L
Coffee, tea (excluding black tea), herbal and fruit infusions, chicory; tea, 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	9.6 g/L[b]
Food supplements as defined in Directive 2002/46/EC, excluding food supplements for infants (EC, 2002)	3.0 g/day for general population 1.2 g/day for young children
Uses Authorised Under Commission Implementing Decision 2017/2201 (EU, 2017)	
Infant formulae as defined in Directive 2006/141/EC (EC, 2006a)and 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
Follow-on formulae as defined in Directive 2006/141/EC (EC, 2006a) and 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

2'-FL = 2'-*O*-fucosyllactose; UHT = ultra-high temperature

[a] From 20 July 2016 the category 'Bread and pasta products for people intolerant to gluten as defined in Regulation (EC) No 41/2009' shall be replaced by the following: 'Bread and pasta products bearing statements on the absence or reduced presence of gluten in accordance with the requirements of Commission Implementing Regulation (EU) No 828/2014 (EC, 2009; EU, 2014).

[b] The maximum level refers to the products ready to use.

The authorised uses and maximum levels for LNnT are stipulated in Annex II of *Commission Implementing Decision (EU) 2016/375 of 11 March 2016 authorising the placing on the market of lacto-*

N-neotetraose as a novel food ingredient under Regulation (EC) No 258/97 of the European Parliament and of the Council, as replicated in Table D.5.1-2 below

Table D.5.1-2 Authorised Uses of LNnT in the EU as *per* Commission Implementing Decision 2016/375

Food Category	Maximum Levels
Unflavoured pasteurised and sterilised (including UHT) milk-based products	0.6 g/l
Unflavoured fermented milk-based products	0.6 g/l for beverages 9.6 g/kg for products other than beverages
Flavoured fermented milk-based products including heat-treated products	0.6 g/l for beverages 9.6 g/kg for products other than beverages
Dairy analogues, including beverage whiteners	0.6 g/l for beverages 6 g/kg for products other than beverages 200 g/kg for whitener
Cereal bars	6 g/kg
Table-top sweeteners	100 g/kg
Infant formulae as defined in Directive 2006/141/EC (EC, 2006a)	0.6 g/l in combination with 1.2 g/l of 2'-*O*-fucosyllactose at a ratio of 1:2 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
Follow-on formulae as defined in Directive 2006/141/EC (EC, 2006a)	0.6 g/l in combination with 1.2 g/l of 2'-*O*-fucosyllactose at a ratio of 1:2 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
Processed cereal-based food and baby food for infants and young children as defined in Directive 2006/125/EC (EC, 2006b)	6 g/kg for products other than beverages 0.6 g/l for liquid food ready for use, marketed as such or reconstituted as instructed by the manufacturer
Milk-based drinks and similar products intended for young children	0.6 g/l for milk-based drinks and similar products added alone or in combination with 2'-*O*-fucosyllactose, at concentrations 1.2 g/l, at a ratio of 1:2 in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
Dietary foods for special medical purposes as defined in Directive 1999/21/EC (EC, 1999)	In accordance with the particular nutritional requirements of the persons for whom the products are intended
Foods intended for use in energy-restricted diets for weight reduction as defined in Directive 96/8/EC (only for products presented as a replacement for the whole of the daily diet) (EC, 1996)	2.4 g/l for drinks 20 g/kg for bars
Bread and pasta products for people intolerant to gluten as defined in Regulation (EC) No 41/2009 (EC, 2009)[a]	30 g/kg
Flavoured drinks	0.6 g/l
Coffee, tea (excluding black tea), herbal and fruit infusions, chicory; tea, 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	4.8 g/l[b]
Food supplements as defined in Directive 2002/46/EC, excluding food supplements for infants (EC, 2002)	1.5 g/day for general population 0.6 g/day for young children

LNnT = Lacto-*N*-neotetraose; UHT = ultra-high temperature.

[a] From 20 July 2016 the category "Foodstuffs for people intolerant to gluten as defined in Regulation (EC) No 41/2009" shall be replaced by the following: "Foodstuffs bearing statements on the absence or reduced presence of gluten in accordance with the requirements of Commission Implementing Regulation (EU) No 828/2014 (EU, 2014)".

[b] The maximum level refers to the products ready to use.

D.5.2 United States

The food uses and maximum use levels of Glycom's 2'-FL obtained by fermentation that are considered GRAS in the U.S. is presented in Table D.5.2-1 below. These are similar to the food uses and maximum

use levels that are concluded to be GRAS for Glycom's chemically-synthesised 2'-FL ingredient (see GRN 546 for details) (U.S. FDA, 2015a). Additionally, 2'-FL from fermentation by Jennewein has been concluded GRAS for use in term infant formula and toddler formula (as consumed) at levels of up to 2,000 mg 2'-FL per L (GRN 571) (U.S. FDA, 2015b). All GRAS notifications have received a "no questions" response from the FDA.

Table D.5.2-1 Individual Food Uses and Use Levels for 2'-FL from Microbial Fermentation that are GRAS in the U.S. (GRN 650) (U.S. FDA, 2016a)

Food Category	Food-Uses	RACC[a]	Maximum Use Level (g/RACC)	Maximum Use Level (g/kg or g/L)[b]
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	1.2	5
	Sports, Isotonic, and Energy Drinks	240 mL	0.28	1.2
Dairy Product Analogues	Imitation Milks	240 mL	0.28	1.2
	Non-Dairy Yogurt	225 g	1.2	5.3
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.24	2.4
	Toddler Formulas	100 mL[c]	0.24	2.4
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.084 to 2.04	12
	Other Drinks for Young Children	120 mL	0.14	1.2
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	30 g	1.2	40
Milk, Whole and Skim	Unflavoured Pasteurised and Sterilised milk[d]	240 mL	0.28	1.2
Milk Products	Buttermilk	240 mL	0.28	1.2
	Flavoured Milk	240 mL	0.28	1.2
	Milk-Based Meal Replacement Beverages, for Weight Reduction	240 mL	1.2	5
	Yogurt	225 g	1.2	5.3
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.28	1.2

2'-FL = 2'-*O*-fucosyllactose; RACC = Reference Amounts Customarily Consumed; U.S. = United States.
[a] Serving sizes were based on RACCs per Eating Occasion in the United States Code of Federal Regulations (21 CFR §101.12 - U.S. FDA, 2016d).
[b] The proposed maximum use level is presented on a g/kg basis for solids and on a g/L basis for liquids.
[c] RACC not available, 100 mL employed as an approximation.
[d] Milk is a standardised food in the United States. When the milk is fortified with 2'-FL, it will then be classified as a milk product.

The food uses and maximum use levels of Glycom's LNnT obtained by fermentation that are concluded GRAS in the U.S. is presented in Table D.5.2-2. These are similar to the food uses and maximum use levels that are concluded to be GRAS for Glycom's chemically-synthesised LNnT ingredient (see GRN 547 for details) (U.S. FDA, 2015c). Both GRAS notifications have received a "no questions" response from the FDA.

Table D.5.2-2 Individual Food Uses and Use Levels for LNnT Produced by Microbial Fermentation that are GRAS in the U.S. (GRN 659) (U.S. FDA, 2016b)

Food Category	Food-Uses	RACC[a]	Use Level (g/RACC)	Maximum Use Level (g/kg or g/L)[b]
Beverages and Beverage Bases	Meal Replacement Drinks, for Weight Reduction	240 mL	0.6	2.5
	Sports, Isotonic, and Energy Drinks	240 mL	0.14	0.58
Dairy Product Analogues	Imitation Milks	240 mL	0.14	0.58
	Non-Dairy Yogurt	225 g	0.6	2.67
Infant and Toddler Foods	Term Infant Formulas	100 mL[c]	0.06	0.60
	Toddler Formulas	100 mL[c]	0.06	0.60
	Other Baby Foods for Infants and Young Children	7 to 170 g	0.02 to 0.68	3.0
	Other Drinks for Young Children	120 mL	0.07	0.58
Grain Products and Pastas	Meal Replacement Bars, for Weight Reduction	30 g	0.6	20.0
Milk, Whole and Skim	Unflavoured Pasteurised and Sterilised milk[d]	240 mL	0.14	0.58
Milk Products	Buttermilk	240 mL	0.14	0.58
	Flavoured Milk	240 mL	0.14	0.58
	Milk-Based Meal Replacement Drinks, for Weight Reduction	240 mL	0.6	2.5
	Yogurt	225 g	0.6	2.67
Processed Fruits and Fruit Juices	Fruit Juices and Nectars	240 mL	0.14	0.58

LNnT = lacto-*N*-neotetraose; RACC = Reference Amounts Customarily Consumed; U.S. = United States.
[a] Serving sizes were based on Reference Amounts Customarily Consumed (RACC) per Eating Occasion in the U.S. CFR (21 CFR §101.12 - U.S. FDA, 2016d).
[b] The maximum use level is presented on a g/kg basis for solids and on a g/L basis for liquids.
[c] RACC not available, 100 mL employed as an approximation.
[d] Milk is a standardised food in the United States. When the milk is fortified with LNnT it will then be classified as a milk product.

D.5.3 Other Jurisdictions

An overview of the uses and use levels for 2'-FL and LNnT that has been accepted in other jurisdictions are summarised in Table D.5.3-1 below.

Table D.5.3-1 Accepted Uses of 2'-FL and LNnT in Other Jurisdictions

Jurisdiction	Ingredient	Accepted Food Uses	Maximum Use Level in g/L (As Consumed)
Israel	2'-FL by fermentation (Jennewein)	Milk-based infant formula (0 to 6 months)	2.0
		Milk-based follow-on formula (6 to 12 months)	2.0
Singapore	2'-FL by fermentation (Glycom)[a]	Infant formula, including follow-on formula (0 to 12 months)	1.2
		Growing-up milks (12 to 36 months)	1.2
	LNnT by fermentation (Glycom)[a]	Infant formula, including follow-on formula (0 to 12 months)	0.6
		Growing-up milks (12 to 36 months)	0.6

2'-FL = 2'-*O*-fucosyllactose; LNnT = lacto-*N*-neotetraose
[a] In Singapore, 2'-FL and LNnT may be added either singly or in combination to the food uses listed here.

D.6 Estimated Intake of 2'-FL from Proposed Uses

D.6.1 Australia/New Zealand

As part of FSANZ's Proposal P306 for the addition of inulin/FOS and GOS to Food (FSANZ, 2008), a theoretical estimate of formula intake was prepared for infants aged 3, 9, and 12 months of age, as well as children age 1 to 3 years. Using the theoretical estimate of formula intake presented in this document, together with the 50th percentiles of body weight, an estimate of the intake of 2'-FL and LNnT from their proposed uses in Australia/New Zealand was derived (Table D.6.1-1). The level of intake of 2'-FL and LNnT from these uses are within the ranges that have been reported from its presence in human breast milk (see Section C.1).

Table D.6.1-1 Estimated Intake of 2'-FL and LNnT from their Proposed Uses Based on Theoretical Intakes of Infant Formula Products and Formulated Supplementary Foods for Young Children in Australia/New Zealand

Age Group	50th Percentile of Body Weight (kg)	Estimated Consumption of Formula (mL/day)	Estimated Intake of 2'-FL (Use Level of 1.2 g/L)		Estimated Intake of LNnT (Use Level of 0.6 g/L)	
			g/day	mg/kg body weight/day	g/day	mg/kg body weight/day
3 Months	6.4	800	0.96	150	0.48	75
9 Months	8.9	545	0.65	73	0.33	37
1 Year	9.6	425	0.51	53	0.26	27
1 to 3 Years	9.6	280	0.34	35	0.17	18

2'-FL = 2'-*O*-fucosyllactose.

Similar estimated levels of intake were derived assuming that 2'-FL and LNnT will be added to 2 representative brands of infant formula, follow-on formula, and formulated supplementary foods for young children (*i.e.*, milk products intended for children age 1 to 3 years old) that are marketed in Australia/New Zealand. The estimated intakes of 2'-FL and LNnT were derived using the typical recommended intake levels of these formula products, as summarised in Table D.6.1-2. Again, the level of intake of 2'-FL and LNnT estimated from these uses are within the ranges that have been reported from its presence in human breast milk (see Section C.1). Additionally, it should be highlighted that the estimated intake levels were derived using the *maximum proposed use levels* of 2'-FL (*i.e.*, 1.2 g/L in the reconstituted product) and LNnT (*i.e.*, 0.6 g/L in the reconstituted product), and the actual inclusion rate may be less when it is formulated into products.

Table D.6.1-2 Estimated Intake of 2'-FL and LNnT from their Proposed Uses Based on Recommended Intake Levels of Infant Formula Products and Formulated Supplementary Foods for Young Children Marketed in Australia and New Zealand

Product Category	Estimated Intake of the Product			Estimated Intake of 2'-FL (g/day)[a]	Estimated Intake of LNnT (g/day)[a]
	# of Servings per Day	Volume (mL) per Serving	Total Volume (mL) Consumed per Day		
Representative Brand A (Australia)					
Infant Formula	5	200[b]	1000	1.2	0.6
Follow-on Formula	3 to 4	230	690 to 920	0.8 to 1.1	0.4 to 0.6
Toddler milk[c]	1 to 2	230	230 to 460	0.3 to 0.6	0.1 to 0.3
Representative Brand B (New Zealand)					
Infant Formula	5	200[b]	1000	1.2	0.6
Follow-on Formula	4 to 5	200	800 to 1000	1.0 to 1.2	0.5 to 0.6
Toddler milk[c]	1 to 2	220	220 to 440	0.3 to 0.5	0.1 to 0.3

2'-FL = 2'-*O*-fucosyllactose; LNnT = lacto-*N*-neotetraose

[a] Assuming that 2'-FL is added at levels of 1.2 g/L of the reconstituted product, and LNnT is added at levels of 0.6 g/L of the reconstituted product.

[b] Infant formula has graduated feeding volumes according to the age of the infant. This volume is intended for infants age 3 to 4 months, as recommended per the feeding table of the manufacturer.

[c] Toddler milks are products that are classified as formulated supplementary foods for young children (Standard 2.9.3, Division 4).

D.6.2 Exposure Assessments that were Previously Conducted

Dietary exposure assessment has been conducted to estimate the intake to 2'-FL and LNnT from its intended uses in the EU and U.S., which includes broader range of food categories, such as foods for infants and young children, as well as conventional foods that are intended for the general population (see Section D.5). As 2'-FL and LNnT are intended for use in a larger number of food categories in these other jurisdictions, the estimated daily intake will likely be higher than the levels anticipated from the proposed uses of 2'-FL and LNnT in Australia/New Zealand, which will be limited to infant formula, follow-on formula, and formulated supplementary foods for young children. Even so, a brief summary of the results of these evaluations is provided below as an indicator for the expected dietary exposures of these ingredients in other countries.

D.6.2.1 European Union

To estimate the dietary intake of 2'-FL and LNnT from their intended uses in the EU, food consumption data from the United Kingdom (UK) was used as a surrogate. Data from this assessment, as replicated from the EFSA Scientific Opinion on the safety of 2'-FL and LNnT as a novel food ingredient (EFSA, 2015a,b), are presented below in Tables D.6.2.1-1 to D.6.2.1-4.

Table D.6.2.1-1 Estimated Daily Intake of 2'-FL from All Authorised Food Categories[a] in the EU by Population Group

Population Group	Age Group	Total n	General Population Intake (g/day)		Consumer Only Intake (g/day)			
			Mean	95th Percentile	%	n	Mean	95th Percentile
Infants and Young Children (DNSIYC Data, 2011)								
Infants	4 to 6 months	329	2.70	5.45	98.1	323	2.75	5.49
Infants	7 to 12 months	1,319	2.44	5.98	94.8	1,252	2.58	6.12
Young Children	13 to 17 months	1,035	0.87	3.34	67.5	688	1.28	3.80
Toddlers and Children (UK NDNS Data, 2008-2010)								
Toddlers	1 to 3 years	219	1.67	3.41	100.0	219	1.67	3.41

2'-FL = 2'-*O*-fucosyllactose; DNSIYC = Diet and Nutrition Survey on Infants and Young Children; NDNS = National Diet and Nutrition Survey; UK = United Kingdom.

[a] For the DNSIYC, only intakes based on Category 13.1 'Foods for infants and young children' (13.1.1, 13.1.2, 13.1.3 and 13.1.4) were included in the assessment.

Table D.6.2.1-2 Estimated Daily Intake of 2'-FL on a Body Weight Basis from All Authorised Food Categories[a] in the EU by Population Group

Population Group	Age Group (months)	Total n	General Population Intake (mg/kg bw/day)		Consumer Only Intake (mg/kg bw/day)			
			Mean	95th Percentile	%	n	Mean	95th Percentile
Infants and Young Children (DNSIYC Data, 2011)								
Infants	4 to 6	329	332	666	98.1	323	338	668
Infants	7 to 12	1,319	259	636	94.8	1,252	273	641
Young Children	13 to 17	1,035	79	316	67.5	688	118	355
Toddlers and Children (UK NDNS Data, 2008-2010)								
Toddlers	1 to 3 years	219	120	247	100.0	219	120	247

2'-FL = 2'-*O*-fucosyllactose; bw = body weight; DNSIYC = Diet and Nutrition Survey on Infants and Young Children; NDNS = National Diet and Nutrition Survey; UK = United Kingdom.

[a] For the DNSIYC, only intakes based on Category 13.1 'Foods for infants and young children' (13.1.1, 13.1.2, 13.1.3 and 13.1.4) were included in the assessment

Table D.6.2.1-3 Estimated Daily Intake of LNnT from All Authorised Food Categories[a] in the EU by Population Group

Population Group	Age Group (months)	Total n	General Population Intake (g/day)		Consumer Only Intake (g/day)			
			Mean	95th Percentile	%	n	Mean	95th Percentile
Infants and Young Children (DNSIYC Data, 2011)								
Infants	4 to 6	329	1.08	2.69	98.1	323	1.11	2.75
Infants	7 to 12	1,319	1.01	2.75	94.8	1,252	1.07	2.77
Young Children	13 to 17	1,035	0.37	1.54	67.5	688	0.54	1.73
Toddlers and Children (UK NDNS Data, 2008-2010)								
Toddlers	1 to 3 years	219	0.89	1.90	100.0	219	0.89	1.90

DNSIYC = Diet and Nutrition Survey on Infants and Young Children; LNnT = lacto-*N*-neotetraose; NDNS = National Diet and Nutrition Survey; UK = United Kingdom.
[a] For the DNSIYC, only intakes based on Category 13.1 'Foods for infants and young children' (13.1.1, 13.1.2, 13.1.3 and 13.1.4) were included in the assessment

Table D.6.2.1-4 Estimated Daily Intake of LNnT on a Body Weight Basis from All Proposed Food Categories[a] in the EU by Population Group

Population Group	Age Group (months)	Total n	General Population Intake (mg/kg bw/day)		Consumer Only Intake (mg/kg bw/day)			
			Mean	95th Percentile	%	n	Mean	95th Percentile
Infants and Young Children (DNSIYC Data, 2011)								
Infants	4 to 6	329	133	328	98.1	323	135	329
Infants	7 to 12	1,319	107	293	94.8	1,252	113	295
Young Children	13 to 17	1,035	34	142	67.5	688	50	159
Toddlers and Children (UK NDNS Data, 2008-2010)								
Toddlers	1 to 3 years	219	63	132	100.0	219	63	132

bw = body weight; DNSIYC = Diet and Nutrition Survey on Infants and Young Children; LNnT = lacto-*N*-neotetraose; UK = United Kingdom.
[a] For the DNSIYC, only intakes based on Category 13.1 'Foods for infants and young children' (13.1.1, 13.1.2, 13.1.3 and 13.1.4) were included in the assessment.

D.6.2.2 United States

The estimated daily intake of 2'-FL and LNnT based on their intended uses in the U.S. are published in the GRAS notices (GRNs 546; 547; 650; 659) (U.S. FDA, 2015a,c, 2016a,b). Data from this assessment are presented below in Tables D.6.2.2-1 to D.6.2.2-4.

Table D.6.2.2-1 Estimated Daily Intake of 2'-FL from All GRAS Food Uses in the U.S.

Population Group	Age Group	*Per Capita* Intake (g/day)		Consumers Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Estimates in Infants (2009-2010 NHANES Data; GRN 546) (U.S. FDA, 2015a)							
Infants	0 to 6 months	2.36	5.11	80.5	168	2.93	5.29
Infants	7 to 12 months	4.63	8.36	100.0	161	4.63	8.36
Estimates in Toddlers and Children (2011-2012 NHANES Data; GRN 650) (U.S. FDA, 2016a)							
Toddlers	1 to 3 years	1.11	1.96	99.3	561	1.12	1.97

2'-FL = 2'-*O*-fucosyllactose; GRAS = Generally Recognized as Safe; GRN = GRAS Registry Number; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

Table D.6.2.2-2 Estimated Daily Intake of 2'-FL on a Body Weight Basis from All GRAS Food Uses in the U.S.

Population Group	Age Group (Years)	*Per Capita* Intake (mg/kg bw/day)		Consumers Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Estimates in Infants (2009-2010 NHANES Data; GRN 546) (U.S. FDA, 2015a)							
Infants	0 to 6 months	362.1	681.7	80.5	168	449.7	712.4
Infants	7 to 12 months	520.2	987.1	100.0	161	520.2	987.1
Estimates in Toddlers and Children (2011-2012 NHANES Data; GRN 650) (U.S. FDA, 2016a)							
Toddlers	1 to 3 years	84.4	146.0	99.3	558	84.9	146.0

2'-FL = 2'-*O*-fucosyllactose; bw = body weight; GRAS = Generally Recognized as Safe; GRN = GRAS Registry Number; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

Table D.6.2.2-3 Estimated Daily Intake of LNnT from All GRAS Food Uses in the U.S.

Population Group	Age Group	*Per Capita* Intake (g/day)		Consumer Only Intake (g/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Estimates in Infants (2009-2010 NHANES Data; GRN 547) (U.S. FDA, 2015c)							
Infants	0 to 6 months	0.66	1.56	80.5	168	0.82	1.60
Infants	7 to 12 months	1.50	2.69	100	161	1.50	2.69
Estimates in Toddlers and Children (2011-2012 NHANES Data; GRN 659) (U.S. FDA, 2016b)							
Toddlers	1 to 3 years	0.51	0.90	99.3	561	0.51	0.90

GRAS = Generally Recognized as Safe; GRN = GRAS Registry Number; LNnT = lacto-*N*-neotetraose; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

Table D.6.2.2-4 Estimated Daily Intake of LNnT on a Body Weight Basis from All GRAS Food Uses in the U.S.

Population Group	Age Group (Years)	*Per Capita* Intake (mg/kg bw/day)		Consumer Only Intake (mg/kg bw/day)			
		Mean	90th Percentile	%	n	Mean	90th Percentile
Estimates in Infants (2009-2010 NHANES Data; GRN 547) (U.S. FDA, 2015c)							
Infants	0 to 6 months	100.2	203.3	80.5	168	124.5	208.5
Infants	7 to 12 months	169.5	312.9	100	161	169.5	312.9
Estimates in Toddlers and Children (2011-2012 NHANES Data; GRN 659) (U.S. FDA, 2016b)							

Table D.6.2.2-4 Estimated Daily Intake of LNnT on a Body Weight Basis from All GRAS Food Uses in the U.S.

Toddlers	1 to 3 years	38.1	67.7	99.3	558	38.4	67.7

bw = body weight; GRAS = Generally Recognized as Safe; GRN = GRAS Registry Number; LNnT = lacto-*N*-neotetraose; NHANES = National Health and Nutrition Examination Survey; U.S. = United States.

E. INFORMATION ON THE NUTRITIONAL AND HEALTH IMPACT (PURPOSE AND EFFICACY) OF THE NOVEL FOOD

The nutritional and health impact of Glycom's 2'-FL and LNnT ingredients, including the rationale and data to support the beneficial physiological effects from their addition to infant formula products and formulated supplementary foods for young children, are described in this Section. Specifically, this Section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel Foods), Guideline 3.6.2 (Special Purpose Food – Infant Formula Products), and Guideline 3.6.3 (Special Purpose Foods – Other Foods) of the Food Standards Australia New Zealand Application Handbook (FSANZ, 2016). The corresponding Sections of this Application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	**Required Information Described in the Guideline**	**Section(s) of the Application where this is Addressed**
Guideline 3.5.2 – Novel Foods	***E. Information on the nutritional and health impact of the novel food***	
	E.1. Information to demonstrate that the use of the novel food or novel food ingredient will not cause a nutritional imbalance in the diet.	Section E.3
	E.2 Information to demonstrate that the addition of the novel food ingredient will not create a significant negative public health impact	Section E.4
Guideline 3.6.2 – Special Purpose Food (Infant Formula Products)	***A. Information related to composition***	
	A.1 Purpose of the compositional change	Section E.1
	A.2 Data for supporting evidence	Section E.2; Section C
	A.3 Specific information requirements for the nutritional safety, tolerance and efficacy of the proposed compositional change (specifically subsection A.3.1) a) Characterisation of proposed substance or the comparable substances in breast milk	Section E.2; Section C.1
	b) Nutritional safety and tolerance of the proposed compositional change	Section C
	c) Efficacy of the proposed compositional change	Section E.2
Guideline 3.6.3 – Special Purpose Food (Other foods)	***A. Information related to general compositional requirements***	
	A.1 Information on the identity and physical and physiological need of the target population	Section E.1
	A.2 Purpose of the compositional change	Section E.1
	A.3 Information related to the safety of the proposed compositional change	Section C
	A.4 Information related to the nutritional impact or performance impact of the proposed compositional change	Section E.2

E.1 Purpose of the Compositional Change

E.1.1 Addition to Infant Formula Products

It is well established that human breastmilk is the optimal form of nourishment for infants during the first 6 months of life, and should continue upon introduction to complementary foods up to 2 years (NHMRC, 2012; WHO, 2017). However, if breastfeeding is not possible or if the mother chooses not to breastfeed, commercially available infant formulas are considered to be a suitable substitute for human breast milk. Considering that breast-feeding has been shown to provide a wide range of beneficial health effects to the infant in comparison to formula feeding (Gale and Martyn, 1996; Hanson, 2007; Horta *et al.*, 2007; Agostoni *et al.*, 2009; Iacovou and Sevilla-Sanz, 2010; Brion *et al.,* 2011; Deoni *et al.*, 2013; Morrow and Chantry, 2013; Quigley *et al.*, 2016; Victora *et al.*, 2016), there have been much efforts over time to refine the composition of infant formula to improve its suitability and to better reflect the composition of breastmilk (Carver, 2003; Thompkinson and Kharb, 2007; Stevens *et al.*, 2009; EFSA, 2014c; Green Corkins and Shurley, 2016). Yet, even today with much progress and control over the composition of infant formula, human breastmilk is still considered to be the "gold standard" for infant nutrition.

Currently, the majority of infant formula and follow-on formula on the market is formulated with mature cow's milk as a base. The biggest compositional difference between these infant formula products and human breast milk is that the latter contains a unique fraction of structurally diverse, non-digestible oligosaccharides which are not present in mature cow milk to any significant degree. A large number of such HMOs have been identified; the wealth of analytical data on the quantities of individual HMOs clearly shows that a subset of merely 10 to 15 individual structures makes up the large majority of the biomass (*i.e*., more than ~85%) (see Appendix III-b). 2'-FL is on average the most abundant HMO; it is reported to comprise up to 15 to 20% (w/w) of the total HMO biomass (Castanys-Muñoz *et al.*, 2013). In pooled samples of human breastmilk, levels of 2'-FL have been reported to range from 1.0 to 8.4 g/L in colostrum, 2.1 to 2.8 g/L in transition milk, and from 0.7 to 3.9 g/L in mature milk (see Section C.1; Appendix III-b). LNnT is also one of the 10 most abundant HMOs in breast milk, accounting for approximately 2 to 4% of the total HMO biomass (see Appendix III-b). Levels of LNnT have been reported to range from 0.2 to 0.5 g/L in colostrum, 0.2 to 0.6 g/L in transitional milk, and 0.04 to 1.1 g/L in mature milk (see Section C.1; Appendix III-b). Additional details on the types and levels of HMOs in human breast milk are presented in Appendix III-a.

Therefore, the addition of 2'-FL at levels of up to 1.2 g/L, either alone or in combination with up to 600 mg/L of LNnT, to infant formula and follow-on formula will result in the development of human milk substitutes that better reflect the compositional profile of oligosaccharides of breast milk. This is consistent with principles set forth by the Australia and New Zealand Food Regulation Ministerial Council's Policy Guideline on the Regulation of Infant Formula Products, which states that "*the composition of breastmilk should be used as a primary reference for determining the composition of infant formula and follow-on formula*". This is similarly highlighted in the Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (Codex Stan 72 - Codex Alimentarius, 2007), which states "*ingredients may be added to formula in order to provide substances ordinarily found in milk and to ensure that the formulation is suitable as a sole source of nutrition for the infant or to provide other benefits that are similar to outcomes of populations of breastfed babies.*" (Article 3.2.1). The proposed use of Glycom's 2'-FL ingredient in infant formula and follow-on formula at maximum levels of 1.2 g/L, either alone or in combination with up to 600 mg/L of LNnT, is well within the natural ranges that have been observed for these HMOs in human milk samples (see Section C.1). As such, the addition of 2'-FL and LNnT to infant formula products is supported on a teleological basis by allowing for the development of infant formula products that more closely match the composition of human milk. The inclusion of 2'-FL and LNnT as a source of human-identical milk oligosaccharides also provides a distinct advantage over supplementation of infant formula products with currently approved

non-digestible oligosaccharides such as inulin-type fructans and galacto-oligosaccharides, which are not naturally present in human breast milk to any significant degree.

E.1.2 Addition to Formulated Supplementary Foods for Young Children

In addition to infant formula products, 2'-FL is proposed for inclusion in formulated supplementary foods for young children at levels of up to 1.2 g/L, either alone or in combination with up to 600 mg/L of LNnT. The target population of these products is healthy young children 1 to 3 years of age (*i.e*., "toddlers"), and 2'-FL (either alone or in combination with LNnT) will be mainly added to formulated milk products that are specifically intended for use by these children (*i.e.*, "toddler milks"). Young children are at a life stage that is a natural progression from infancy; they have increasing levels of physical activity and demands for energy and nutrient intakes as compared to the infant, which are satisfied by a gradually diversified and varied diet. Even so, as young children learn to adapt to the family diet, some may exhibit 'fussy eating' behaviours or food rejections, which could lead to instances where nutrient and energy intakes may not be sufficient. Therefore, formulated supplementary foods (*e.g*., milk products) targeted towards this subgroup may be warranted. The addition of 2'-FL, either on its own or together with LNnT, to formulated supplementary foods for young children would help increase the intake of these non-digestible oligosaccharides from the diet.

It should be noted that the addition of HiMOs, including 2'-FL and LNnT, to formulated milk products for young children can also be supported on a teleological basis, as it is consistent with efforts to produce milk products that more closely match the nutrient composition of human milk. During historical times, durations of breastfeeding were generally longer than what occurs in western society today (Canadian Paediatric Society, 2004). When "natural weaning" is practiced (*i.e.,* driven by cues that the child is ready to stop breastfeeding), complete weaning usually takes place between two and four years of age (Canadian Paediatric Society, 2004). Even now, current global public health advice from the World Health Organization is for young children to continue to be breastfed to "2 years and beyond" (WHO, 2017). Similarly, the evidence-based Infant Feeding Guidelines by the National Health and Medical Research Council (NHMRC) recommends that "breastfeeding is continued until 12 months of age and beyond, for as long as the mother and child desire" (NHMRC, 2012). Some limited Australian data (Magarey *et al*., 2016) suggest that 20 to 25% of infants that have just entered toddlerhood (*i.e.*, 1-year old) are still receiving some breast milk in their diet. Therefore, young children who are still breastfeeding will continue to receive HMOs as a normal part of their diet. For young children where breastfeeding has ceased, formulated milk products containing 2'-FL (either alone or together with LNnT) can provide a means for continued exposure to the likely beneficial effects of these non-digestible oligosaccharides (see Section E.3).

E.2 Nutritional and Performance Impact (Efficacy) of the Proposed Compositional Change

E.2.1 Overview

The abundance of HMOs in human breast milk suggests that they may play important physiological roles for the developing child. This hypothesis is supported by the finding that these oligosaccharides do not occur anywhere else in nature but mammalian milk, and that human milk is significantly more complex in its oligosaccharide composition and content than any other mammalian milk investigated to date (Warren *et al.*, 2001; Urashima *et al*., 2001). Such compositional differences are particularly pronounced when human milk is compared to milk from domestic farm animals, such as cows (Nakamura and Urashima, 2004). Furthermore, the distribution of characteristic structural features (*i.e.*, absence or presence of certain sugars or carbohydrate epitopes) in different mammalian milks oftentimes do not cluster according to phylogeny (Bishop and Gagneux, 2007), not even within the order of primates (Tao *et al.*, 2011). This finding strongly suggests that the carbohydrate epitopes

presented by milk oligosaccharides present an evolutionary hotspot reflecting an arms race between host and pathogens (Springer and Gagneux, 2016). Finally, there is increasing evidence to support the hypothesis that HMOs and the human microbiota co-evolved over millions of years to optimise outcomes for the infant under the constraint of the "mother-offspring conflict" (Urashima *et al.*, 2012; Moeller *et al.,* 2016; Springer and Gagneux, 2016; Yamada *et al.*, 2017). Therefore, there are compelling arguments that support a fundamental biological role of HMOs in human milk from an evolutionary perspective. Given the unique presence and abundance of HMOs in human milk, much research focus has been made to empirically understand the physiological roles of HMOs, particularly 2'-FL and LNnT, and the mechanisms through which they may act.

As described in Section C.1, HMOs including 2'-FL and LNnT contain glycosidic linkages that are resistant to hydrolysis by human digestive enzymes (Brand-Miller *et al.*, 1995, 1998; Engfer *et al*., 2000; Gnoth *et al*., 2000). Therefore, they are expected to largely escape digestion in the upper gastrointestinal tract and reach the colon intact, where they may serve as growth substrates for the commensal microflora present. It is well recognised that HMOs can facilitate the selective proliferation of beneficial gut bacteria; accordingly, they are often referred to in the literature as having "prebiotic" properties. Although the term "prebiotic" is not currently defined in the Australia New Zealand Food Standards Code, it is generally understood by the industry and the scientific community to mean: "*a substrate that is selectively utilized by host microorganisms conferring a health benefit*" (Gibson *et al.*, 2017). The ability of HMOs to promote the growth of a beneficial gut bacteria was discussed in a recent Scientific Opinion on the essential composition of infant- and follow-on formula published by EFSA (EFSA, 2014c), in which the following was stated:

> "*The third main component in human milk after lactose and fat are neutral and acid*[ic] *oligosaccharides in concentrations between around 5-15 g/L (Aggett et al., 2003; Coppa et al., 2011). The structure of about 200 human milk oligosaccharides has been identified (Kunz et al., 2000). These oligosaccharides are typically composed of 3–23 monosaccharide units, including glucose, galactose, N-acetylglucosamine, fucose and sialic acid. Approximately 20 oligosaccharides make up more than 90 % of the total amount of oligosaccharides in human milk, with the principal oligosaccharides being fucosyllactoses, lacto-N-tetraose, lacto-N-neotetraose, sialyllactoses, lacto-N-fucopentaoses (I–V) and lacto-N-difucohexaoses (I–III). The neutral linear and branched-chain oligosaccharides are fucosylated to a varying degree and make up 80-85 % of the total amount of oligosaccharides in human milk, whereas the acidic oligosaccharides contain sialic acid and make up 15-20 % of the total amount. The production of oligosaccharides is genetically determined and the individual pattern of oligosaccharides differs between women (Ninonuevo et al., 2006)."*
>
> ***The oligosaccharides of human milk are considered to be one of the principal growth factors, for example, for bifidobacteria in the infant gut and are responsible for the composition of the gut microbiota found in breast-fed infants****. The fermentation of non-digestible oligosaccharides leads to the generation of organic acids (lactic acid) and short-chain fatty acids (SCFAs) such as acetic, propionic and butyric acids. Butyrate is a main source of energy for the colonocytes and has effects on cell differentiation. Acetate and propionate are absorbed from the colon and thus provide energy to the host (Aggett et al., 2003).*"

Furthermore, FSANZ has also referred to the "prebiotic" effects of HMOs. For example, in FSANZ's Proposal to amend the Code, which included provisions to allow for the addition of inulin-derived substances and GOS to infant formula products, infant foods, and formulated supplementary foods for young children (Proposal P306; FSANZ, 2008), it is stated that: "*Human breast milk oligosaccharides are recognised prebiotics, because of their resistance to digestion by host enzymes and their ability to selectively promote the growth of Bifidobacterium and Lactobacillus in the colon (Coppa et al., 2004a)*".

The biological roles of HMOs have been investigated from the moment of their discovery in the 1950's [reviewed in (Kunz, 2012)]. In fact, the discovery of HMOs was directly connected to their bifidogenic effect, since they were first identified as the predominant "*bifidus factor*" of human milk (György, 1953; Gauhe *et al.*, 1954; György *et al.*, 1954a,b; György and Rose, 1955). However, owing to the complex composition of the total HMO fraction (and intricate molecular structure of individual components), it was for a very long time impossible to isolate or produce significant amounts of HMOs allowing for larger scale study of their explicit nutritional effects. This circumstance has even been highlighted in the latest EFSA opinion on the essential composition of infant- and follow-on formula (EFSA, 2014c) where it states in Chapter 5.4.5.3 on page 35:

> "*Because of the variety, variability, complexity and polymorphism of human milk oligosaccharides, the addition to IF and FOF of a mixture of oligosaccharides mimicking those found in breast milk is not feasible and oligosaccharides which are currently added to IF and FOF are not comparable to human milk oligosaccharides. Instead, oligofructosyl-saccharose (oligofructose; FOS) and oligogalactosyl-lactose (oligogalactose; GOS) have been used in IF and FOF. FOS is not found in human milk and GOS is found only in trace amounts.*"

However, despite the principal verity of the above EFSA comment, there already exists a huge body of basic research studies (including receptor binding studies, single cell and cell culture studies, and animal studies) that have been performed using either the whole HMO fraction isolated from human milk, or individually isolated and purified HMOs like 2'-FL and LNnT. Furthermore, data to support that intake of HMOs are associated with beneficial effects are available from observational studies examining the relationship between levels of HMOs (specifically 2'-FL and LNnT) in breastmilk or Secretor status with health outcomes in infants, as well as from clinical intervention studies whereby formulas containing these HiMOs were administered under a controlled setting. The purported roles of HMOs, particularly 2'-FL and LNnT, and the mechanisms behind their main physiological effects, are briefly summarised below in Sections E.2.2 and E.2.3. Additional details are further provided in Appendix III-a.

E.2.2 Data from *In Vitro* and Animal Studies

A) Bifidogenic Properties

Human breastmilk is known to exhibit bifidogenic effects, promoting the proliferation of specific bacterial strains such as *Bifidobacterium infantis*, *B. breve* and *B. bifidum* (Bezirtzoglou *et al.*, 2011). Bifidobacteria are an important component of the commensal microflora; they contribute to the maintenance of intestinal health, and by extension, promotes various beneficial effects on human health (Picard *et al.*, 2005; Hidalgo-Cantabrana *et al.*, 2017). Since *Bifidobacterium* is abundant in the microbiota of breast-fed infants, their acquisition and role in HMO metabolism have drawn a lot of research attention in recent years.

The bifidogenic effects of 2'-FL, either alone or in combination with LNnT, have been demonstrated through a number of *in vitro* and animal studies (see Appendix III-a), as well as from human observational data and intervention studies (see Section E.2.3). Certain *Bifidobacterium* species in the intestines express the genes encoding enzymes that allow for the utilisation of HMOs. For example, 2'-FL is hydrolysed to fucose and lactose by α-1,2-fucosidases. These enzymes are rare in the bacterial domain of life but have been identified in common infant gut commensals like *B. infantis* (Kim *et al.*, 2013) and *B. bifidum* (Katayama *et al.*, 2004, 2008; Nagae *et al.*, 2007). LNnT, on the other hand, is hydrolysed to galactose and lacto-*N*-triose II by specific β-1,4-galactosidases, and then further to *N*-acetyl-glucosamine (GlcNAc) and lactose by N-acetyl-β-D-hexosaminidases. These enzymes are equally rare in the bacterial domain, but have also been identified in *B. infantis* (Yoshida *et al.*, 2012; Garrido *et al.*, 2012) and *B. bifidum* (Miwa *et al.*, 2010). The rarity and specificity of the metabolic adaptions required to utilise 2'-FL and LNnT is exemplified by recent studies demonstrating that even within bacterial species that are known to metabolise 2'-FL and LNnT, most strains lack the respective enzymes

(Thongaram *et al.*, 2017). Thus, the ability to metabolise 2'-FL and LNnT is not only species-specific, but rather strain-specific. Further discussion of the data to support that *Bifidobacterium* species express the enzymes needed to utilise 2'-FL and LNnT are described in Appendix III-a.

Hence, due to their specific genes that co-evolved with humans to utilise HMOs (Moeller *et al.*, 2016; Segre and Salafsky, 2016), *Bifidobacterium* species in the infant's intestine have a growth advantage when compared to other members of the intestinal microbial community (Sela *et al.*, 2008; Yu *et al.*, 2013; Hoeflinger *et al.*, 2015). By selectively promoting the growth of bifidobacteria, HMOs such as 2'-FL and LNnT are expected to reduce the colonisation of pathogenic species through several postulated mechanisms. Some bifidobacterial species produce bacteriocins, proteinaceous toxins which inhibit the growth of unfavourable bacteria (Collado *et al.*, 2005; Yildirim and Johnson, 1998). Additionally, the generation of metabolites such as short-chain fatty acids (SCFAs) from the fermentation of 2'-FL and LNnT by the colonic microflora (*e.g.*, *B. infantis*, *B. breve* and *B. bifidum*) will reduce colonic pH, thereby creating an unfavourable environment for the growth of pathogenic species. The SCFA metabolites are also important in the metabolic "cross-feeding" and function of other important bacterial species within the colonic microflora (see Appendix III-a for further details). The presence of bifidogenic species is also expected to inhibit the colonisation of potentially pathogenic strains due to the competition for nutrients and adhesion sites in the intestines (see Appendix III-a).

B) Anti-Infective Effect Against Pathogens

It has been suggested that HMOs, including 2'-FL and LNnT, may have anti-infective effects against a range of pathogens, based on 3 principle mechanisms:

1) Inhibition of the growth and colonisation of pathogens by providing a competitive advantage to non-pathogenic commensals such as bifidobacteria (through the mechanisms described in the section above);

2) Competitive binding to the carbohydrate recognition domains of pathogen-generated proteins, e.g. surface proteins and/or toxins [reviewed by (Zopf and Roth, 1996; Sharon and Ofek, 2000; Newburg, 2009; Newburg *et al.*, 2005; Kunz and Rudloff, 2006; Hickey, 2012; Bode, 2015; El-Hawiet *et al.*, 2015)];

3) Modulation of the immune response either locally in the intestine or systemically (Abrahamsson and Sherman, 2014; He *et al.*, 2014; Goehring *et al.*, 2016; Yu *et al.*, 2016).

Many viral, bacterial pathogens or toxins need to adhere to mucosal surfaces to colonise or invade the host and cause disease (Bode 2012, 2015). Most pathogens express binding proteins (lectins) that bind to glycans on the host's epithelial cell surface (Springer and Gagneux, 2016), particularly the carbohydrate histo-blood group and lewis antigens (Heggelund *et al.*, 2017; Rodríguez-Díaz *et al.*, 2017). Some HMOs are structurally similar to these intestinal epithelial cell surface glycans, and thus serve as decoy receptors to prevent pathogen binding and enhance pathogen clearance (Simon *et al*., 1997; Gustafsson *et al*., 2006). This beneficial effect of HMOs is highly dependent on their structure. In case of 2'-FL and LNnT, selective binding has been demonstrated for a range of pathogen lectins, including those from rotaviruses (Ramani *et al.*, 2016), noroviruses (Koromyslova *et al.*, 2017), *Campylobacter jejuni* (Lane *et al.*, 2011), and *Pseudomonas aeruginosa* (Ramphal *et al.*, 1991). Furthermore, a range of *in vitro* competitive binding assays conducted with 2'-FL and LNnT individually confirm that these HMOs may inhibit the adhesion of pathogens (see Appendix III-a). The results of several *in vitro* binding assays, adhesion assays in various cell lines, and a suckling mouse lethality assay also suggest that 2'-FL and LNnT can individually compete with binding of pathogen-derived toxins (*e.g.*, enterotoxins). These results are further supported by animal studies where the anti-infective effects of 2'-FL and LNnT have been demonstrated (see Appendix III-a). For example, Yu *et al.* (2016) reported that 2'-FL reduced *C. jejuni* colonisation in mice, and also improved histologic features of intestinal inflammation and induced inflammatory signalling molecules of acute-phase mucosal immune response. Similarly, Li *et al.*

(2014) reported that a mixture of HMOs containing 40% 2'-FL and 35% LNnT (plus 10% 6'-SL, 5% 3'-SL and 10% free NANA) reduced the duration of rotavirus-induced diarrhoea in piglets, and that the protective effect was related to the ability of HMOs to influence the host immunity by stimulating a balanced Th1 and Th2 cytokine response (Li *et al.*, 2014). A follow-up study by the same researchers provided insight into the possible mechanism of action that underlies partial protection against rotavirus-infection by the HMO mixture and showed that the same effect was not elicited by other non-digestible oligosaccharides (*i.e.*, short-chain galacto-oligosaccharides and long-chain fructo-oligosaccharides) (Comstock *et al.*, 2017; Donovan, 2017). There are also some data from humans to suggest that the intake of HMOs (including 2'-FL and LNnT) are associated with a reduced likelihood of infections (see Section E.2.3).

C) Intestinal Barrier Function and Immune Modulation

The intestinal barrier function plays an important role in maintaining host health. Intestinal barrier dysfunction plays a role in infant diseases such as inflammatory bowel disease, infectious enteritis, and necrotising enterocolitis (NEC). Several studies have shown that human breast milk decreases the intestinal permeability and therefore enhances the physical barrier of an infant intestine (Halpern and Denning, 2015). The protective effect of HMOs on gut barrier function and immune modulation has been examined using both *in vitro* and animal models (see Appendix III-a). The ability of 2'-FL and LNnT to support the intestinal barrier function and modulate the immune system can be explained through both indirect and direct mechanisms (for a recent review, please see Donovan and Comstock, 2016). Indirectly, changes in the microbiota composition may affect the infant's immune system, leading to protection against pathogens. It has been shown that stimulating the growth of infant-dominant bifidobacteria by HMOs can enhance tight junction protein expression and immunomodulatory IL-10 in CaCo2 cells and intestinal epithelial cells; however, this effect was not observed when the bacteria grew on lactose (Chichlowski *et al.*, 2012). HMOs may also directly affect gut barrier function, as demonstrated in a study by Holscher *et al.* (2014) where LNnT was found to increase transepithelial resistance in CaCo2 cells. Additionally, LNnT has been reported to reduce intestinal crypt cell proliferation and apoptosis (Hester and Donovan, 2012). In a mouse model for small bowel syndrome, Mezoff *et al.* reported that 2'-FL changed the intestinal microbial community and affected characteristic histological changes such as increase in crypt depth and villus height. In addition, the study showed that administration of 2'-FL (indirectly or directly) initiated an upregulation in genes related to mucosal immune response (Mezoff *et al.*, 2016).

The immunomodulatory properties of HMOs such 2'-FL and LNnT is related to their structures. HMOs are highly similar in structure to other human glycans, presenting many identical epitopes. Lectins are carbohydrate-binding proteins on the surfaces of mammalian cells that translate recognition of specific carbohydrate motifs and the spatial presentation of those motifs into action. The intestinal dendritic cells (DC) present in the organised lymphoid structures of the intestinal immune system are implicated both in the maintenance of tolerance, and in the generation of protective immune responses against pathogens. This flexibility in function is due to the ability of accurately sense their local environment and use these signals to shape the nature of the ensuing immune response. One class of lectins, called C-type lectins, found amongst others on the surface of intestinal DC can use fucosylated glycans as ligands; hence the interaction between C-type lectins on intestinal DC and fucosylated HMOs, such as 2'-FL, can contribute to immunity and immune tolerance (Donovan and Comstock, 2016). This interaction is assumed to be the mechanism behind the results reported by Castillo-Courtade *et al.* (2015), where dietary 2'-FL was shown to attenuate food allergy symptoms and help establish oral tolerance by inducing IL-10^{+} regulatory cells and stabilising mast cells in an animal model.

Galectins are another group of soluble carbohydrate-binding proteins with immunomodulatory properties that act either intracellularly or extracellularly. Galectins bind predominantly glycosphingolipids of the lacto- and neolacto-series, including poly-*N*-acetyl-lactosamine (poly-Galβ1,4GlcNAc), but have also been explicitly shown to use LNnT as efficient ligand (Collins *et al.*, 2014;

Halimi *et al.*, 2014; Bohari *et al.*, 2016; Noll *et al.*, 2016; Shams-Ud-Doha *et al.,* 2017). Recently, efficient and selective binding of 2'-FL to 4 human galectins was also determined (Shams-Ud-Doha *et al.,* 2017). Galectins plays a distinct role in the control of immune cell homeostasis by affecting the differentiation of regulatory DCs, which promotes T cell tolerance through mechanisms involving IL-27 and IL-10; hence, the interaction between galectins and HMOs, such as LNnT and 2'-FL, is presumed to contribute to immunity and immune tolerance (Davicino *et al.*, 2011; Rabinovich *et al.*, 2012).

It is notable that HMOs may act either locally on cells of the mucosa-associated lymphoid tissues, or on a systemic level (Bode, 2012). As explained in Section C.1, a small fraction of the ingested HMOs can be absorbed in the gastrointestinal tract and reach systemic circulation. The absorption rate of HMOs from the gastrointestinal tract has been estimated to be approximately 1% of the total HMO intake, thus their systemic levels are estimated in the range of 10 to 100 mg/mL (Bode *et al.*, 2004). This concentration is sufficient to directly affect and activate immune cells circulating in the blood. Several *in vitro* and *ex vivo* experiments have shown that 2'-FL and LNnT can individually interact with immune cells affecting immune cell activation and cytokine production (Velupillai and Harn, 1994; Zhu *et al.*, 2003; Zhu *et al.*, 2005; Amin *et al.*, 2008; Rabquer *et al.*, 2012; Comstock *et al.*, 2014) (see Appendix III-a).

E.2.3 Data from Human Observational Studies and Clinical Trials

A) Observational Studies

As mentioned, breast-feeding has been shown to provide a wide range of beneficial health effects to the infant in comparison to formula feeding, and there is evidence to suggest that these benefits may be attributed to the presence of HMOs in breastmilk. 2'-FL and LNnT are amongst the most abundant HMOs in human breastmilk. The levels of individual HMOs in breastmilk (including 2'-FL and LNnT) have been positively associated with health outcomes in infants (see Appendix III-a). Furthermore, the mother's Secretor status (*i.e.*, expresses the α-1,2-fucosyltransferase enzyme in their mammary glands) has also been associated with various beneficial health outcomes. For example, infants of Secretor mothers who express 2'-FL as the predominant milk oligosaccharide in their breastmilk had a more favourable intestinal microflora profile, as well as a reduced risk for allergy (Sprenger *et al.,* 2017b) and diarrhoea (Morrow *et al.,* 2004), when compared to infants of non-secretor mothers who lack 2'-FL in their breastmilk. Further details of these studies are provided in Appendix III-a.

B) Clinical Trials Conducted in Infants

A randomised, blinded, controlled, multi-centre, parallel-design study has been conducted where infants were administered formula containing up to 1.2 g 2'-FL together with up to 0.6 g LNnT [27] for the first 4 months of life. Another group of infants received a control formula without these HiMOs. The infants were exclusively fed the test or control formulas for the first 4 months of age. Complementary foods were allowed to be introduced beginning at 4 months of age. At 6 months of age, the infants in both study groups (test and control formula) were switched to an intact protein, cow's milk–based, follow-up formula without HiMOs for feedings through to 12 months of age. Both the 2'-FL and LNnT ingredients used in this study were produced by Glycom. Additional details of this study, including the outcomes of the safety-related parameters assessed, have been provided in Section C.4.1. Although the primary objective of this study was to investigate the safety and tolerability of 2'-FL and LNnT (Puccio *et al.*, 2017), the secondary endpoints collected provides evidence that supplementation with 2'-FL and LNnT may have beneficial health outcomes. Infants receiving the formula supplemented with HiMOs had

[27] The targeted concentration in the infant formula tested in this study was 1.0 to 1.2 g/L of 2'-FL and 0.5 to 0.6 g/L of LNnT. Actual trial product was within this range and in all cases above the 1.0 and 0.5 g/L threshold, respectively: the test formula was analyzed to be 1.04 to 1.14 g/L (SD 0.073 to 0.08 g/L) for 2'-FL and 0.52 to 0.61 g/L (SD 0.028 to 0.033 g/L) for LNnT.

significantly fewer parental reports of bronchitis, lower respiratory tract infections, antipyretic use, and antibiotic use, when compared to infants receiving the control formula (Puccio *et al.,* 2017).

In the same study, stool samples were collected at 3 months of age for assessment of microbiota using 16S rRNA gene sequencing and metagenomics; metabolic signature was also examined using proton NMR-based metabolite profiling (Alliet *et al.*, 2016; Steenhout *et al*., 2016). Stool samples that were collected from a sample of breast-fed infants in the same study served as a reference control. Infants fed formula containing the 2 HiMOs experienced a shift in their faecal microbiota profile towards one that more closely resemble those of breast-fed infants. The global average microbial composition for the sub-group of infants with stool samples that followed the study protocol showed similar pattern between control (n=65) and test (n=58) at the genus level, although samples obtained from infants receiving the test formula were closer to breastfed (n=34) than control samples. Calculations of microbial alpha diversity and comparison of the global microbiota composition confirmed that test was different from control at the genus level ($p<0.001$) and closer to the breastfed reference. Statistical analysis (corrected for false discovery rate) identified several taxa differentially present in control and test including *Bifidobacterium* (p=0.01), *Escherichia* (p=0.008) and unclassified *Coprobacillaceae* (p=0.01). Multivariate analysis identified several influential metabolites that discriminated between test, control and breastfed groups including phenylalanine, isoleucine, tyrosine, faecal organic acids and fucosylated compounds. The values observed for the test formula group were more similar to those observed in the breast-fed group compared with control, a finding that suggests reduced protein fermentation. The study authors concluded that: "*Together, the stool microbiota and metabolic signature show that the addition of 2'FL and LNnT to a starter infant formula shift the stool microbiota towards that observed in breastfed infants, both in composition and function.*"

Another randomised, controlled study has been conducted to investigate the safety of 2'-FL, in which infants were administered one of the following 3 formulas for 4 months: i) a standard, milk-based, commercially available infant formula containing 2.4 g GOS/L (control formula); ii) the standard formula supplemented with 0.2 g 2'-FL/L and 2.2 g GOS/L; or iii) the standard infant formula supplemented with 1.0 g 2'-FL/L and 1.4 g GOS/L (Marriage *et al.*, 2015; Goehring *et al.*, 2016). A comparator (reference) group comprised of infants consuming human milk (by breast and/or bottle) was also included. Additional details of this study, including the outcomes of the safety-related parameters assessed, have been provided in Section C.4.1. The outcomes related to biomarkers of immune function are described in a publication by Goehring *et al.* (2016). At 6 weeks of age, 2 to 3 mL of non-fasting venous blood was drawn and analysed for immunophenotypic markers (by flow cytometry) for the following cell surface markers: CD4, CD8, CD20, and CD56. Plasma samples were analysed for cytokines: IFN-α2, IFN-δ, IL-10, IL-1 receptor antagonist (IL-1ra), IL-1α, IL-1β, IL-6, IFN-δ–induced protein 10, RANTES (regulated upon activation, normal T cell expressed and secreted), and TNF-α. RNA from peripheral blood mononuclear cells were quantified and used to detect a respiratory syncytial virus (RSV)-specific gene product, NS1, to quantify viral load.

The control formula group exhibited lower percentages of circulating T lymphocytes and CD8+ lymphocytes compared to the breastfed group; however, no significant differences in any cell type were observed between the 2'-FL supplemented groups and the control and breastfed groups with the exception of a lower CD8+ population in infants receiving 1.0 g 2'-FL/L compared to the breastfed group. The inflammatory cytokine profiles revealed a statistically significant higher concentration of circulating inflammatory cytokines IFN-α2, IL-1β, IL-6, TNF-α and IL1ra in the control formula group when compared to be breastfed group. However, no statistically significant differences were observed in the groups receiving the experimental formulas containing the 2 different doses of 2'-FL, when compared to the breastfed group. No significant differences in the other plasma cytokines or RANTES were observed between any of the groups. Furthermore, no significant differences in RSV NS1 viral load were observed between any groups. In *ex vivo* RSV-induced peripheral blood mononuclear cells (PMBC) culture, cytokine production in the breastfed group did not significantly differ from the groups receiving the formulas containing 2'-FL; however, TNF-α and IFN-γ were significantly lower, and a non-significant

trend towards reduced IL-1ra and IL-6 was observed, in the breastfed group compared to the control formula. The study authors concluded that infants provided 2'-FL fortified formulas exhibited lower plasma and *ex vivo* inflammatory cytokine profiles, similar to those of a breastfed reference group. In contrast, such effects were not observed among infants administered the control formula containing GOS only.

C) Clinical Trial Conducted in Adults

A randomised, placebo-controlled, double-blind, parallel-design study has been conducted whereby various doses of 2'-FL and LNnT, either individually or combined, were administered to healthy adult volunteers for 2 weeks (Elison *et al.*, 2016). A comparator control group receiving glucose as a placebo was also included. An overview of the different dose combinations that were tested in this study is presented in Table E.2.3-1 below. The test articles containing the allocated amount of 2'-FL and/or LNnT were provided in powder form, and the participants were instructed to dissolve the powder in approximately 250 mL of water prior to intake in the morning with breakfast. Additional details on the study methods, as well as outcomes related to safety and tolerability, have been described in Section C.4.2.

Table E.2.3-1 Doses Administered in the Clinical Trial Conducted in Healthy Adults (Elison *et al.*, 2016)

Group No.	Daily Dose of 2'-FL (grams)	Daily Dose of LNnT (grams)
1	20	0
2	10	0
3	5	0
4	0	20
5	0	10
6	0	5
7	13.33	6.67
8	6.67	3.33
9	3.33	1.67
Control	2 grams Dextropure (glucose)	

2'-FL = 2'-*O*-fucosyllactose; LNnT = lacto-N-neotetraose.

In addition to the safety and tolerability parameters, the effects of 2'-FL and/or LNnT administration on the gut microbiota were also assessed. Four faecal samples were collected from each subject; each sample was collected at approximately 1-week intervals and they were subjected to microbiota profiling using 16S rRNA sequencing. Samples 1 and 2 were collected before the start of the intervention, and the average of the analysed values in these 2 samples served as the baseline levels. Samples 3 and 4 were collected during the intervention period, and an average of the analysis from these samples represented the values observed as a result of the intervention. A statistically significant increase in the relative abundance of Actinobacteria from baseline was observed in individuals consuming 5 or 10 g/day of 2'-FL; 5, 10 or 20 g/day of LNnT; and 10 or 20 g/day of the mixture of 2'-FL and LNnT provided at a 2:1 ratio. Administration of 2'-FL and/or LNnT also resulted in a reduction in the relative abundance of Firmicutes and Proteobacteria; the latter phylum includes the *Enterobacteriaceae* family, some members of which are pathobionts. The increase in Actinobacteria can be fully accounted for by an increase in *Bifidobacterium*. Compared to the placebo, a significant increase in the abundance of *Bifidobacterium* was observed in individuals consuming 10 g/day of 2'-FL; 5, 10 or 20 g/day of LNnT; and 10 or 20 g/day of the mixture of 2'-FL and LNnT provided at a 2:1 ratio.

Overall, the results of this study provide evidence that 2'-FL and LNnT, when administered either alone or in combination, mediate favourable changes in the gut microbiota composition through their bifidogenic effects.

E.2.4 Summary

The totality of data summarised in Section E.2.2 and E.2.3 demonstrate that the proposed uses of 2'-FL, either alone or in combination with LNnT, will serve to benefit the infants and young children consuming products containing these HiMOs [*i.e.*, infant formula, follow-on formula, formulated supplementary foods (milks) for young children]. Evidence from studies conducted using *in vitro* and in animal models demonstrate that HMOs, including 2'-FL and LNnT, can exert beneficial health effects through their favourable modulation of the gut microflora profile, protection against infections by pathogens, strengthening of the intestinal barrier function, and immunomodulatory functions (see Appendix III-a for further details). As described in Section E.2.3 and Appendix III-a, the levels of 2'-FL and LNnT in breastmilk, or Secretor status, has been associated with positive health outcomes in infants. Moreover, infants consuming formula supplemented with 2'-FL in combination with LNnT experience a shift of the faecal microbiota profile towards one that more closely resemble those of breast-fed infants (Alliet *et al.*, 2016; Steenhout *et al.*, 2016). The administration of formula containing 2'-FL resulted in plasma levels of inflammatory cytokines that more closely mimic those in breastmilk-fed infants, whereas no such effects were observed in infants administered a control formula that contained only GOS (Goehring *et al.*, 2016).

Although clinical studies have not been conducted to investigate the effects of 2'-FL and LNnT supplementation on young children (age 1 to 3 years), it is anticipated that the beneficial effects of 2'-FL, either alone or in combination with LNnT, can be further extrapolated to this population group. As these HiMOs are non-digestible oligosaccharides, they are expected to favourably modify the gut microflora in a similar manner as those that have been observed in the clinical studies conducted in infants (Alliet *et al.*, 2016; Steenhout *et al.*, 2016), and as supported by the *in vitro* and animal studies. Young children are at a life stage that is a natural progression from infancy, and there is no reason to suggest that the mechanism by which 2'-FL and LNnT exerts these effects would differ according to age. The data from the clinical study involving adults where 2'-FL and LNnT, either alone or in combination, produced favourable changes to the microbiota profile provides further support that such beneficial effects would also be similarly observed among young children, given that the microbiota start to fully resemble those of an adult in terms of composition and diversity by 2 to 5 years of age (Rodríguez *et al.,* 2015). E.3 Information to Demonstrate that the Proposed Uses of 2'-FL and LNnT Will Not Cause a Nutritional Imbalance in the Diet

The addition of 2'-FL, either alone or in combination with LNnT, to infant formula and follow-on formula is at a level similar to (and no higher than) the levels occurring in breastmilk. As mentioned in Section E.1, the inclusion of 2' FL (with or without LNnT) is to solely bring available infant formula products closer in composition to human breast milk, in line with principles that have been established by the Australia/New Zealand Ministerial Policy Guideline on the Regulation of Infant Formula Products, and in the Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (Codex Stan 72 - Codex Alimentarius, 2007). These HiMOs are also proposed for addition to formulated supplementary foods for young children, which will complement the range of other non-digestible oligosaccharides that are already permitted for inclusion to these types of products in Australia/New Zealand (*i.e*., inulin-type fructans and galacto-oligosaccharides).

Infant formula products, as well as formulated supplementary foods for young children, that contain 2'-FL (with or without LNnT) would be required to meet the compositional requirements that have been already established in Standard 2.9.1 and 2.9.3, and thus would be nutritionally balanced. No anti-nutritional effects (*i.e*., reductions in the availability of nutrients) are expected following the consumption of these HiMOs, and clinical studies have been conducted in both infants and adults that demonstrate 2'-FL (with or without LNnT) to be safe, well tolerated, and supported appropriate growth (see Section C.4). As such, no adverse effects on overall diet quality are anticipated based on the proposed uses of 2'-FL, when used alone or in combination with LNnT.

E.4 Information to Demonstrate that the Proposed Uses of 2'-FL and LNnT Will Not Create a Negative Public Health Impact

2'-FL and LNnT obtained from fermentation are not expected to create a negative impact on public health when used as an ingredient in infant formula, follow-on formula, and formulated supplementary foods for young children. Both ingredients are highly purified; the 2'-FL preparation is specified to contain not less than 94.0% of 2'-FL and not less than 96.0% of HiMS, while the LNnT preparation is specified to contain not less than 92% LNnT and not less than 95.0% of HiMS. As described in Section C, the safety of 2'-FL and LNnT has been well demonstrated through a number of preclinical toxicology studies as well as clinical studies conducted in both infants and adults. Furthermore, as described in Section E.3 above, 2'-FL and LNnT are also not expected to have any other negative nutritional impact, such as adversely altering the nutritional profile of foods or the bioavailability of other nutrients. Rather, foods containing the ingredient are expected to have the health benefits associated with non-digestible oligosaccharides, such as improvements in gut health and function, which will have positive effects on the consumers (*e.g.,* by providing more options for healthier foods) and the industry (*e.g.,* increased product innovation) alike.

F. INFORMATION RELATED TO POTENTIAL IMPACT ON CONSUMER UNDERSTANDING AND BEHAVIOUR

The potential impact on consumer understanding and behaviour in relation to products containing Glycom's 2'-FL and LNnT ingredients is discussed in this Section. Specifically, this Section is completed in accordance with the information requirements outlined in the relevant sections of Guideline 3.5.2 (Novel Foods), Guideline 3.6.2 (Special Purpose Food – Infant Formula Products), and Guideline 3.6.3 (Special Purpose Foods – Other Foods) of the Food Standards Australia New Zealand Application Handbook (FSANZ, 2016). The corresponding Sections of this Application in which the information requirements have been addressed are summarised in the table below.

Relevant Guideline	**Required Information Described in the Guideline**	**Section(s) of the Application where this is Addressed**
Guideline 3.5.2 – Novel Foods	F.1 Information to demonstrate the level of consumer awareness and understanding of the novel food or novel food ingredient	Section F.1
	F.2 Information on the actual or potential behaviour of consumers in response to the novel food or novel food ingredient	Section F.2
	F.3 Information to demonstrate that the food(s) containing the novel food ingredient will not adversely affect any population groups	Section F.3
Guideline 3.6.2 – Special Purpose Food (Infant Formula Products)	***C. Information related to labelling requirements under Part 2.9 of the Code***	
	C.1 Information related to safety or nutritional impact of the proposed labelling change	Section F.1, F.3
	C.2 Information to demonstrate that the proposed labelling change will be understood and will assist consumers	Section F.2
	D. Information related to internationally recognised standards, codes of practice, recommendations AND guidelines	Section F.1
Guideline 3.6.3 – Special Purpose Food (Other foods)	***C. Information related to labelling requirements under Part 2.9 of the Code***	
	C.1 Information related to safety or nutritional impact of the proposed labelling change	Section F.1, F.3
	C.2 Information to demonstrate that the proposed labelling change will be understood and will assist consumers, if applicable	Section F.2
	D. Information related to internationally recognised codes of practice and guidelines	Section F.1

F.1 Information to Demonstrate Consumer Awareness and Understanding of the Novel Food Ingredient

In addition to the general labelling requirements established under Part 1.2 of the Code, infant formula products (*i.e.*, infant formula, follow-on formula) containing 2'-FL and LNnT will be labelled in accordance with the specific provisions established in the Standard 2.9.1 of the Code. The presence of 2'-FL and LNnT in infant formula products will be made known by their declaration on the ingredient list. In other countries (*e.g.*, Spain) where infant formula containing 2'-FL and LNnT have been sold, these HiMOs have been declared as "2'-O-Fucosyllactose (2'-FL)" and "Lacto-N-neotetraose (LNnT)" in the ingredient list, along with a statement that "human milk oligosaccharides are the third most abundant component in human milk". As the purpose of adding 2'-FL and LNnT to infant formula products is to make its composition closer to that of human breast milk, which is considered the gold-standard of infant feeding, it is anticipated that such products may be recommended by health care professionals and in turn will be well-received by consumers. Industry will inform and educate the health care professionals about the novel food ingredient. Of note, no claim statements are currently being made for 2'-FL or LNnT in infant formula products that are already on the market in other jurisdictions.

Formulated supplementary foods for young children containing 2'-FL and LNnT will also meet all of the labelling provisions provided for in Standard 2.9.3, and as the general labelling requirements established under Part 1.2 of the Code. Similar to infant formula products, the presence of 2'-FL and LNnT will be declared as "2'-O-Fucosyllactose (2'-FL)" and "Lacto-N-neotetraose (LNnT)" in the ingredient list of formulated supplementary foods for young children. The addition of 2'-FL and LNnT will be similar to those of other non-digestible oligosaccharide ingredients (*i.e.*, inulin-type fructans and galacto-oligosaccharides) that are already permitted for inclusion to these types of products in Australia/New Zealand, and thus it is also expected to be well accepted by consumers. At the moment, it is not anticipated that claim statements relating to 2'-FL and LNnT will be made in formulated supplementary foods for young children.

Infant formula products and formulated supplementary foods for young children containing 2'-FL and LNnT will adhere to all other relevant standards or guidelines that are applicable to such products (*e.g.,* WHO International Code of Marketing of Breast-milk Substitutes, Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants, Codex Guidelines for Formulated Supplementary Foods for Older Infants and Young Children, *etc*.).

F.2 Information on Actual/Potential Behaviour of Consumers in Response to the Novel Food Ingredient

2'-FL and LNnT have been authorised for use in infant formula and follow-on formula, as well as a wide range of food products that are intended for use by the general population, in both the U.S. and EU, as described in Sections C.7 and D.5. More recently, 2'-FL and LNnT have also been granted approval for use in infant formula and growing up milks in Singapore, and 2'-FL has been authorised for use in infant formula in Israel (see Sections C.7 and D.5).

As previously mentioned, the authorisation of 2'-FL and LNnT as novel food ingredients in Australia and New Zealand is anticipated to result in new products which will provide a more diverse range of options for consumers. The anticipated behaviour of Australian and New Zealand consumers in response to the market entry of 2'-FL and LNnT is expected to be comparable to that of EU and U.S. residents whom has benefited from the market introduction of these non-digestible oligosaccharides.

F.3 Information to Demonstrate that the Novel Food Ingredient Will Not Adversely Affect Any Population Groups

2'-FL and LNnT produced by microbial fermentation are structurally identical to their naturally occurring counterparts that are present in human breast milk. The proposed use levels in infant formula and follow-on formula are based on levels observed in human milk; therefore, the dietary intake of 2'-FL and LNnT in formula would result in a comparable exposure to that of breastfeeding infants, and accordingly are considered safe based on their history of consumption. If 2'-FL and LNnT are considered safe in infants, who represents a particularly vulnerable population given that infant formula serves as the sole source of nutrition, then these ingredients will certainly be safe for the older infants and young children who consume an increasingly diversified diet.

The safety of 2'-FL and LNnT have been further substantiated by the results of several preclinical toxicological studies and clinical studies (see Section C). The results of a 90-day oral toxicity study conducted with Glycom's 2'-FL obtained by fermentation have demonstrated that no adverse effects are observed following administration of up to 5,000 mg/kg body weight/day (Penard, 2015). This NOAEL of 5,000 mg/kg body weight/day has been corroborated by other studies on 2'-FL produced by others (Jennewein) and on Glycom's chemically synthesised 2'-FL (see Section C). Moreover, 2'-FL was demonstrated to be safe and well tolerated in a neonatal piglet model when formulated in a milk replacer at levels of up to 2 g/L, which corresponds to intakes up to approximately 291.74 mg/kg body weight/day in males and 298.99 mg/kg body weight/day in females, for 20 days (Hanlon and Thorsrud, 2014). Similar to 2'-FL, the NOAEL for Glycom's LNnT obtained by fermentation was determined to be 5,000 mg/kg body weight/day, the highest dose tested (Penard, 2016). This NOAEL value is corroborated by the results of another 90-day oral toxicity study conducted with the chemically-synthesised LNnT ingredient (Coulet *et al.*, 2013).

In clinical studies, administration of formula containing 2'-FL (1.0 to 1.2 g/L) and LNnT (0.5 to 0.6 g/) to infants during the first 4 months of life was well tolerated and supported age-appropriate growth (Puccio *et al.*, 2017). This is further corroborated by other clinical trials conducted in infants where administration of formula containing 2'-FL (0.2 g/L or 1.0 g/L) with other non-digestible oligosaccharides (GOS, scFOS) was demonstrated to be safe and well tolerated (Marriage *et al.*, 2015; Kajzer *et al.*, 2016). Administration of 2'-FL alone (up to 20 g/day), LNnT alone (up to 20 g/day), or mixture of 2'-FL and LNnT at a 2:1 ratio (up to 20 g/day total) to adults as a single daily dose for 2 weeks was also shown to be safe and well-tolerated (Elison *et al.*, 2016).

It is worth mentioning that the inclusion of 2'-FL (either alone or in combination with LNnT) in infant formula products and formulated supplementary foods for young children will not adversely affect behaviour or diet quality, as these products will be required to meet the compositional standards already in place under Standards 2.9.1 and 2.9.3. Furthermore, the 2'-FL and LNnT ingredients and its production process (including all processing aids, raw materials, and unit operations/filtration aids) are certified to be Halal and Kosher, meaning that it would be suitable for use by individuals requiring this type of food handling. As 2'-FL and LNnT are produced from lactose as a starting material, it is possible that residual amounts of lactose may remain (specification limit: not more than 3%; range from analysis on 4 lots of 2'-FL: 0.57 to 0.84%; range from analysis on 4 lots of LNnT: 0.21 to 0.66%). Currently, it cannot be excluded with high confidence that traces of milk protein may remain; thus, the 2'-FL and LNnT ingredients may not be suitable for use in products that are targeted towards the most sensitive group of children with milk allergies. 2'-FL and LNnT will be added only to formula products that are based on cow's milk, and these products will already be labelled to indicate the presence of milk allergens.

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