APPLICATION TO AMEND THE AUSTRALIA NEW ZEALAND FOOD STANDARDS CODE:

2'-FL/DFL, LNT, 6'-SL SODIUM SALT, AND 3'-SL SODIUM SALT PRODUCED USING GENE TECHNOLOGY FOR USE AS NUTRITIVE SUBSTANCES IN INFANT FORMULA PRODUCTS

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Glycom A/S is a wholly owned indirect affiliate of DSM Nutritional Products Ltd, a company with a registered [redacted]

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Application to Amend the Australia New Zealand Food Standards Code: 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3'-SL Sodium Salt Produced using Gene Technology for Use as Nutritive Substances in Infant Formula Products

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Application to Amend the Australia New Zealand Food Standards Code: 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3'-SL Sodium Salt Produced using Gene Technology for Use as Nutritive Substances in Infant Formula Products

INTRODUCTION

This application is for the approval of 4 human-identical milk oligosaccharide (HiMO) products, produced by microbial fermentation using a modified strain of *Escherichia coli* (*E. coli*) K-12, for use as nutritive substances (alone or in combinations) in infant formula products, namely:

1. Mixture of 2'-fucosyllactose (2'-FL) and difucosyllactose (DFL) ("2' FL/DFL");
2. Lacto-*N*-tetraose (LNT);
3. 6'-Sialyllactose (6'-SL) sodium salt; and
4. 3'-Sialyllactose (3'-SL) sodium salt.

2' FL/DFL, LNT, 6'-SL, and 3'-SL are important components of the human milk oligosaccharide (HMO) fraction of human milk, and structural characteristics of HMOs influence their biological role. The above manufactured HiMOs are purified ingredients that are identical in structure to their corresponding HMOs naturally present in human milk. Briefly:

- 2'-FL and DFL are two distinct fucosylated HMOs that are structurally and biologically closely related since DFL is metabolically obtained from 2'-FL by the simple addition of a second fucose unit ("fucosylation"); thus, 2'-FL and DFL are always found together in human milk. 2'-FL and DFL are produced in the same fermentation and are isolated together, to produce the 2'-FL/DFL mixture. Glycom's 2'-FL ingredient produced by microbial fermentation has already been approved for use in infant formula products in Australia and New Zealand (FSANZ, 2021a).

- LNT is a basic neutral HMO that is derived from lactose by the addition of *N*-acetylglucosamine (GlcNAc) and galactose. The LNT tetrasaccharide is a constitutional isomer of lacto-*N*-neotetraose (LNnT), differing only by a β1-3 *versus* β1-4 linkage between galactose and *N*-acetylglucosamine at the non-reducing terminus, respectively. Glycom's LNnT ingredient produced by microbial fermentation has already been approved for use in infant formula products in Australia and New Zealand (FSANZ, 2021a).

- 6'-SL is a sialylated HMO made from glucose, galactose, and *N*-acetylneuraminic acid (NANA, hereinafter also referred to as "sialic acid"). Glucose and galactose comprise the milk sugar lactose, and NANA is an acidic monosaccharide.

- 3'-SL is a sialylated HMO and is a regio-isomer of 6'-SL, differing only by an α2-3 *versus* α2-6 linkage between galactose and sialic acid, respectively. As a result, 3'-SL and 6'-SL share common biological roles.

In 2014, the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) provided its most recent updated Scientific Opinion on the essential composition of infant and follow-on formula (EFSA, 2014). The Opinion still represents one of the most up-to-date internationally accepted reviews of the science around infant feeding. Within this review, under Section 5.4.2.2, *Non-digestible (non-glycemic) carbohydrates in human milk*, it states:

> *"The third main component in human milk after lactose and fat are neutral and acid oligosaccharides in concentrations between around 5 to 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 to 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 to 85% of the total amount of oligosaccharides in human milk, whereas the acidic oligosaccharides contain sialic acid*

and make up 15 to 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-chained 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). Fermentation products, i.e., SCFAs, contribute to the energy content of the diet, but to a lesser extent than glycaemic carbohydrates. Human milk oligosaccharides are not considered in the estimation of the energy content of the milk."

Thus, it can be noted from Section 5.4.2.2 that EFSA has recognised the principal importance of the human milk oligosaccharide fraction for the infant.

Glycom A/S is a Danish food ingredient manufacturer that has developed the technology to manufacture selected HiMOs on an industrial scale. Glycom is striving to manufacture an ever-increasing proportion of the natural HMO fraction of human milk with the goal to match the natural composition as closely as possible.

The first two HiMOs to have been commercialised by Glycom were 2'-FL and LNnT. Both ingredients are approved in Australia and New Zealand as "Food produced using gene technology" (Schedules 26) for use in "Infant formula products" (Standard 2.9.1, Schedule 29) within the Australia New Zealand Food Standards Code ("the Code"). 2'-FL and LNnT have also gained novel food approval in the European Union (EU), have achieved Generally Recognized as Safe (GRAS) status in the United States (U.S.), and have gained authorisation in numerous other markets [including Argentina, Brazil, Israel, Malaysia, Russia, Switzerland, Singapore, Thailand, Taiwan, and the United Kingdom (UK)]. As a result, both ingredients have been commercialised in infant formula products in many countries worldwide.

Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt have been authorised for use as novel foods in the EU (as well as in Switzerland according to the Novel Food Regulation) following positive Scientific Opinions on their safety from EFSA's NDA Panel, and have FDA-notified GRAS status in the U.S, for similar food uses as 2'-FL and LNnT in both jurisdictions. Furthermore, all 4 HiMOs have gained authorization in for use as novel foods in Israel, Singapore, and the UK, while LNT, 6'-SL sodium salt, and 3'-SL sodium salt have been authorised for use as novel foods in Brazil with forthcoming approval for 2'-FL/DFL.

The intended use of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt in Australia and New Zealand is for the same food use as currently authorised for 2'-FL and LNnT, *i.e.,* "Infant formula products". Specifically, 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are intended to be added to infant formula products alone or in combinations providing levels of the specified HiMOs similar to those naturally occurring in human milk.

The 4 HiMO products that are the subject of this application are manufactured by Glycom each using a living cell fermentation process with recombinant derivatives of the *E. coli* K-12 strain as a processing aid, similar to 2'-FL and LNnT. The resulting products are highly purified. During fermentation, each HiMO is secreted extracellularly from the fermentation organism into the culture medium, which allows for them to be easily separated as purified ingredients. No residual production organism or metabolites thereof remains in the final 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt products.

This dossier has been prepared for evaluation in accordance with the following Guidelines in the Food Standards Australia New Zealand (FSANZ) Application Handbook:

- Guideline 3.3.1: General requirements
- Guideline 3.3.3: Substances used for a nutritive purpose
- Guideline 3.5.1: Foods produced using gene technology
- Guideline 3.6.2: Special purpose foods – infant formula products

Information to demonstrate that Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are safe and appropriate for their intended conditions of use are detailed herein.

SUMMARY OF SOUGHT PERMISSIONS

This application seeks to amend the Code to allow 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced using modified strains of *E. coli* K-12 containing genes for their biosynthesis, according to specifications described in this application, for addition to infant formula products. The proposed amendment impacts the following Schedules of the Code:

Schedule 3

See proposed specifications for 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt in Part 3.3.3 'Substances Used for a Nutritive Purpose', Section B.5.1, of the application.

Schedule 26

Food produced using gene technology of microbial origin		
Substance	**Source**	**Condition of Use**
2'-Fucosyllactose/ difucosyllactose	*Escherichia coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori*	Infant formula products
Lacto-*N*-tetraose	*Escherichia coli* K-12 containing the gene for beta-1,3-N-acetylglucosaminyltransferase from *Neisseria meningitides* and the gene for beta-1,3-galactosyltransferase from *Helicobacter pylori*	Infant formula products
6'-Sialyllactose sodium salt	*Escherichia coli* K-12 containing the gene for alpha-2,6-sialyltransferase from *Photobacterium damsela* and CMP-Neu5Ac synthetase, Neu5Ac synthase, *N*-acetylglucosamine-6-phosphatase epimerase from *Campylobacter jejuni*	Infant formula products
3'-Sialyllactose sodium salt	*Escherichia coli* K-12 containing the gene for alpha-2,3-sialyltransferase from *Neisseria meningitides* and CMP-Neu5Ac synthetase, Neu5Ac synthase, *N*-acetylglucosamine-6-phosphatase epimerase from *Campylobacter jejuni*	Infant formula products

Schedule 29

Infant formula products—substances permitted for use as nutritive substances	
Substance	**Maximum amount per 100 KJ**
2'-Fucosyllactose/ difucosyllactose	96 mg
Lacto-*N*-tetraose	32 mg
6'-Sialyllactose sodium salt	16 mg
3'-Sialyllactose sodium salt	8 mg

PART 3.1.1 – GENERAL REQUIREMENTS

B. Applicant Details

Glycom A/S (“Glycom”), is the applicant and manufacturer of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt, the HiMOs that are the subject of this application.

B.1 Contact Information

[redacted]

B.2 Nature of Applicant’s Business

Glycom A/S was founded in 2005, specialising in the development, synthesis, and commercialisation of HiMOs. Consisting of a R&D headquarters and a production facility, it synergistically covers the whole value chain from research, intellectual property creation, development, technology transfer to engineering and production, isolation-purification, product release, and product approval. The company comprises about 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 intellectual property. Since April 2020 Glycom A/S is a wholly owned indirect affiliate of DSM Nutritional Products Ltd, a company with registered address at Wurmisweg 576, 4303 Kaiseraugst, Switzerland.

C. Purpose of the Application

Human milk contains a number of structurally diverse oligosaccharides, termed human milk oligosaccharide (HMOs), which include 2'-FL, DFL, LNT, 6'-SL, and 3'-SL. Glycom has developed methods to manufacture 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt by fermentation with derivatives of *E. coli* strain K-12 containing biosynthetic genes for their production. The HiMOs produced by microbial fermentation are chemically and structurally identical to the same oligosaccharides that are naturally present in human milk.

Previously, FSANZ has issued an approval report for Glycom’s first two HiMOs produced by microbial fermentation, namely 2'-FL and LNnT (FSANZ, 2019a). Subsequently, upon request by the Australia and New Zealand Ministerial Forum on Food Regulation (the Forum), FSANZ issued a review report re-affirming their decision for the approval of the voluntary addition of 2'-FL alone or in combination with LNnT, produced by microbial fermentation, in infant formula products (FSANZ 2020).

As a result of the above, and *via* Amendment No. 198 of the Food Standards Code *via* the Food Standards Gazette (Commonwealth of Australia, 2021), 2'-FL and LNnT were approved into Schedule 26 as “Food produced using gene technology of microbial origin” for use in infant formula products (as defined in Standard 2.9.1), according to identity and purity provisions laid out in Schedule 3 (Section S3—40 and Section S3—41, respectively), and maximum levels of use laid out in Schedule 29 for special purpose foods (Section S29—5).

Similar to 2'-FL and LNnT, Glycom intends to use 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt as ingredients for use in infant formula products to better reflect the compositional profile of oligosaccharides in human milk.

The purpose of this application is to amend Schedule 26 in order to include 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt as "Food produced using gene technology of microbial origin" for use in infant formula products. It is recognised that the approval of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt under the proposed amendment will require consideration and possible changes to the following Standards and their associated Schedules, as relevant:

- Standard 2.9.1: Infant formula products;
- Schedule 3: Identity and purity;
- Schedule 26: Food produced using gene technology (of microbial origin); and
- Schedule 29: Special purposed foods (S29—5, Infant formula products—substances permitted as nutritive substances, specifically).

Information and data presented in this application support the safe and suitable use of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt for its proposed use in infant formula products.

D. Justification for the Application

D.1 Regulatory Impact Information

D.1.1 Functionalities of the Food

Human milk contains a number of structurally diverse oligosaccharides, termed human milk oligosaccharides (HMOs). Glycom has developed the technology to manufacture HMOs, by microbial fermentation, that are structurally and chemically identical to their counterparts that are naturally present in human milk. Commercial infant formulas are considered the only safe and suitable alternative to human milk up to 12 months of age in non-breastfed or partially breastfed infants (NHMRC, 2012). The development of infant formula has historically been based on the composition of cow's milk. A comparison between the macronutrient content of cow milk, infant formula, and human milk (Viverge *et al*., 1990; Michaelsen *et al*., 1994; Newburg and Neubauer, 1995; Hester *et al*., 2012; Bode, 2013; Newburg, 2013; EFSA, 2014; Lönnerdal *et al*., 2017; Xu *et al*., 2017) reveals that the largest remaining compositional discrepancy between infant formulas and human milk today is the oligosaccharide fraction of human milk, as the milk oligosaccharides detected in human milk are not present in mature cow's milk to any significant degree. Therefore, manufactured HMOs are primarily intended for addition to commercial infant formula and follow-on formula ("infant formula products") at use levels representative of naturally occurring HMO concentrations in human milk, with the goal of enabling commercially available infant formula to match the natural composition of human milk as closely as possible.

2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt, manufactured by Glycom, are intended for addition to infant formula products (Standard 2.9.1) alone or in combinations. Maximum proposed use levels of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt for addition to infant formula products, expressed on a HiMO basis, are consistent with mean concentrations of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL in human milk reported in the literature (see Part 3.6.2, Section A.3.1.2). As HMOs are the third most abundant solid component of human milk reaching concentrations of up 25 g/L in human colostrum and up to 20 g/L in mature human milk (Bode, 2012), and are a structurally and biologically diverse group of complex oligosaccharides, the use of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt will allow for infant formula products that more closely reflect the natural composition of human milk.

In its initial "Approval report – Application A1155 2'-FL and LNnT in infant formula and other products*" dated 20 December 2019,* Section 2.5.1—Section 29—2.2.4*,* FSANZ (2019a) concluded:

> *"There are no public health and safety concerns associated with adding the applicant's 2'-FL and LNnT to infant formula products and FSFYC at the levels permitted which are consistent with average levels in mature human milk. The evidence supports the proposed compositional permission. FSANZ concluded that the requested addition of 2'-FL alone or with LNnT demonstrates a favourable health effect and has the potential to confer beneficial health outcomes in infants and young children. The available evidence demonstrates a mechanism for and pathogen binding with an anti-infective effect against invasive Campylobacter jejuni infection and a bifidogenic effect (an increase in the relative abundance of bifidobacteria in the intestinal microflora)".*

This was followed by the "Review – Application A1155 2'-FL and LNnT in infant formula and other products" that was conducted following the request of the Australia and New Zealand Ministerial Forum, dated 05 October 2020,

which reaffirmed this conclusion following an additional independent expert advisory group (IEAG) third party assessment, and stated in Section 4.2.1 (FSANZ, 2020):

> *"FSANZ's review re-affirms the proposed addition to infant formula products and FSFYC [Formulated Supplementary Foods for Young Children] poses no health risk, engenders favourable physiological effects similar to breastfed infants, with outcomes more similar to breastfed infants than infant consuming unsupplemented formula and a possible link to a favourable health outcome through risk reduction, especially for toddlers."*

The safety and benefit of the addition of a five-HMO blend composed of Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt to infant formula has been evaluated in a randomised controlled trial conducted in healthy term infants 7 to 21 days of age at enrolment (Clinical Trial Registry NCT03722550). Results for safety-related outcomes are available through to 12 months of age (Cohen, 2022 [unpublished] – summarised in Part 3.3.3, Section C.2.2), while results for benefit-related outcomes through to 6 months of age have recently been published (Bosheva *et al*., 2022 – summarised in Part 3.3.3, Section A.2.2). In the publication, the study investigators concluded that that consumption of infant formula with up to 2.5 g/L of the the five-HMO blend through to 6 months of age "*supports the development of the intestinal immune system and gut barrier function and shifts the gut microbiome closer to that of breastfed infants with higher bifidobacteria, particularly B. infantis, and lower toxigenic Clostridioides difficile*."

D.1.2 Costs and Benefits of Application

In its initial "Approval report – Application A1155 2'-FL and LNnT in infant formula and other products*" dated 20 December 2019,* Section 2.5.1—Section 29—2.5.1.1 Consideration of costs and benefits*,* FSANZ (2019a) concluded:

> *"FSANZ's assessment is that the direct and indirect benefits that would arise from permitting the voluntary addition of 2'-FL and LNnT in the manner proposed are likely to outweigh the associated costs."*

This was followed by the "Review – Application A1155 2'-FL and LNnT in infant formula and other products" that was conducted following the request of the Australia and New Zealand Ministerial Forum, dated 05 October 2020 (FSANZ, 2020):

> *"FSANZ's assessment is that the direct and indirect benefits that would arise from permitting the voluntary addition of 2'-FL and LNnT in the manner proposed i.e., safe, possible benefits, and labelling requirements, are likely to outweigh the associated costs to the community. Reaffirming the draft variation has immediate benefits to consumers and the Australia New Zealand infant formula manufacturers, it also encourages ongoing innovation to continue the improvement of infant formula products and FSFYC.*
>
> *Whilst re-affirming approval of the draft variation may not, of itself, substantially act to bridge the socioeconomic gap between formula-fed and breastfed infants, the precedent safeguards the economic viability of the substantial R&D investments in this sector which may lead to further formula improvements. Innovations are also key to creating products that are preferred by consumers both domestically and in the competitive international markets. This in turn supports the profitability of the firms and the jobs in the sector. The Australia New Zealand exports of infant formula and FSFYC are economically significant and bring substantial wealth into the countries."*

Since Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced my microbial fermentation are chemically and structurally identical to their respective counterparts that are naturally present in human milk (*i.e.*, 2'-FL, DFL, LNT, 6'-SL, and 3'-SL, respectively), and are intended to be added to infant formula products to more closely emulate the diversity of HMOs in human milk, similar to 2'-FL and LNnT, FSANZ's conclusions for other HiMOs are considered relevant to the 4 HiMO products that are the subject of this application.

D.2 Impact on International Trade

In its initial "Approval report – Application A1155 2'-FL and LNnT in infant formula and other products" *dated 20 December 2019,* Section 2.5.1—Section 29—2.5.1.1 Consideration of costs and benefits*,* FSANZ (2019a) concluded:

"The applicant's 2'-FL and LNnT is permitted for use in infant formula products and FSFYC in some overseas countries including the EU and US. The proposed permission will enable Australian and New Zealand industries to access and use ingredients that are available to their overseas competitors, which may provide trade opportunities."

In the following "Review – Application A1155 2'-FL and LNnT in infant formula and other products" conducted upon the request of the Australia and New Zealand Ministerial Forum, dated 05 October 2020, FSANZ (2020a) concluded:

"Infant formula products with 2'-FL and LNnT are currently approved and used in 69 countries. FSFYC or similar products for young children containing added 2'-FL and LNnT are also available in most of these countries. Permitting the oligosaccharides in Australia and New Zealand supports domestic companies' ability to stay competitive in the global market and to continue product export. The permission improves harmonisation with international regulations, supports cost-effective manufacturing through consistency with overseas regulations, and supports innovation for manufacturers and researchers in Australia and New Zealand."

2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are already authorised for use in infant formula and follow-on formula in the EU (EU, 2019, 2020, 2021a,b), in Switzerland under the Novel Food Regulation through EU authorisation on the Union list (DFI, 2020; FSVO, 2021), and in the UK (EUR-Lex, 2017). Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation are authorised novel foods in Singapore (approval letters in Appendix X – **Confidential Commercial Information**) and Israel (Israel MOH, 2022a,b,c,d), while the latter three of Glycom's HiMOs are authorised novel foods in Brazil[1] (approval letters in Appendix X – **Confidential Commercial Information**). Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation have also obtained GRAS status in the U.S. for use in non-exempt infant formula and toddler formula (GRNs 815, 833, 880, and 881; U.S. FDA, 2019a,b, 2020a,b). Furthermore, all four HiMOs are registered food ingredients for use in infant foods in Russia[2], and have been added to the Therapeutic Goods (permissible Ingredients) Determination in Australia for use as complementary medicine ingredients in listed medicines in Australia[3]. Therefore, FSANZ's conclusions on the impact of 2'-FL and LNnT on economic and trade benefits in Australia and New Zealand are considered equally relevant to 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt.

E. 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 Part 3.3.3 of the application, in which the safety of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt, 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 of Part 3.3.3 of this applcation, in which the full compositional information and nutrient profiles of foods containing 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are described in detail. A discussion of the nutritional and health impact of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt as food ingredients, individually and in combination, is also provided in Section A of Part 3.3.3 of this application.

c) The prevention of misleading or deceptive conduct: Information supporting objective (c) is provided in Section G of Part 3.3.3. of the application, in which the consumer awareness and potential behaviour in

[1] Forthcoming approval for 2'-FL/DFL.
[2] Register of Certificates of State Registration, available at: http://fp.crc.ru/evrazes/?oper=s&type=max&text_prodnm=%EE%EB%E8%E3%EE%F1%E0%F5%E0%F0%E8%E4&text_ff_firm=&text_firmget=&text_firmmade=&text_usearea=&text_gighark=&pdk=on&text_n_state=&text_n_org=&text_n_otdel=&text_n_okp=&text_n_type=%C5&text_n_currnumb=&text_n_char=&text_n_year=&text_serialnumb=.
[3] Most recent Determination (No. 4) 2022, available at: https://www.legislation.gov.au/Details/F2022L01035.

response to 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt 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 01 July 2019), including the following (FSANZ, 2019b):

- Chapter 3.1: General Requirements;
- Chapter 3.3.3: Substances used for a nutritive purpose;
- Chapter 3.5.1: Foods produced using gene technology;
- Chapter 3.6.2: Special purpose foods – Infant formula products

Comprehensive literature searches have previously been conducted as part of regulatory submissions for 2'-FL/DFL, LNT, 6'-SL sodium salt and 3'-SL sodium salt in the U.S. and EU. As indicated in Section C.3 of Part 3.3.3 of this application, the U.S. FDA and EFSA have previously conducted safety assessments on 2'-FL/DFL, LNT, 6'-SL sodium salt and 3'-SL sodium salt, each of which have obtained approvals in these jurisdictions. Glycom has since been monitoring the literature for new, relevant, publications reporting on the safety and benefit of these HiMOs, which have been incorporated in this application.

F. Assessment Procedure

Glycom considers the Major Procedure (Subdivision D of the FSANZ Act) to be the most appropriate assessment procedure for the evaluation of this application to amend the Australia New Zealand Food Standards Code to Permit the Use of 4 HiMO ingredients (2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt) produced using gene technology for use in infant formula products. Other HiMOs (2'-FL and LNnT) produced by microbial fermentation have previously been reviewed by FSANZ (FSANZ, 2021a,b) and have obtained market authorisation into Schedule 26 – Food produced using gene technology; Schedule 3 – Identity and purity; and Schedule 29 – Special purpose foods.

As with the preceding novel food application for 2'-FL and LNnT submitted by Glycom (Application A1155), the safety of the proposed uses of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt is largely based on the fact that maximum proposed use levels are comparable to those which occur naturally on average for 2'-FL, DFL, LNT, 6'-SL, and 3'-SL within human milk. Safety is further corroborated by:

- Preclinical studies conducted with 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt which have been reviewed by authoritative bodies in the EU (EFSA, 2019a,b, 2020a,b; EU, 2019, 2020, 2021a,b), and to which the U.S. FDA has issued a "no questions" response to a GRAS notice submitted for these ingredients (GRNs 815, 833, 880 and 881 – U.S. FDA, 2019a,b, 2020a,b); and
- An infant clinical study that confirmed non-inferior growth in infants fed test infant formulas with added five-HMO blend composed of Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation compared to the control formula without the HiMOs (Cohen, 2022 [unpublished]; Clinical Trial Registry NCT 037225500).

G. 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 (CCI):

- Appendix III – Comparison to Human Milk
- Appendix IV – Stability Testing Results
- Appendix V – Certificates of Analysis
- Appendix VI – Manufacturing Process
- Appendix VII – Internal Methods of Analysis
- Appendix VIII – Unpublished Pre-Clinical Study Reports

- Appendix IX – Unpublished Clinical Study Report
- Appendix X – SFA Authorisation Letters
- Appendix XI – Post-Market Surveillance Data
- Appendix XII – Production Strain Data

The information contained within these appendices is not publicly available and release of these data would be at a commercial disadvantage to Glycom, having invested considerable capital to develop and validate methods of analyses and also to commission the toxicological studies that substantiate the safety of their specific ingredient.

H. Additional Confidential Information

Glycom requests that that contact information (Section B.1 of Part 3.1.1 of the application) and the statutory declaration (Appendix I), both containing personal information, are kept confidential.

I. Exclusive Capturable Commercial Benefit (ECCB)

Glycom is seeking exclusive permission for the use of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced using technology on the basis that they are highly refined products obtained *via* proprietary manufacturing processes. There has also been significant research and investment by Glycom and its partners into the development of these ingredients. It is envisioned that exclusivity will be specific to Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt when they are used as food ingredients and not to finished food products containing the material. In practice, this means that during the exclusivity period, a manufacturer may incorporate 2'-FL/DFL, LNT, 6'-SL sodium salt, and/or 3'-SL sodium salt into their food products only if they obtain the ingredient(s) 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:** Nutritive substance produced using gene technology
>
> **Brand of the Food:** GlyCare™ 2FL/DFL 8001, GlyCare™ LNT 8001, GlyCare™ 6SL 9001, and GlyCare™ 3SL 9001

As such, it is expected 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."

J. International and Other National Standards

J.1 International Standards

Codex Alimentarius (Codex) International Food Standards do not currently exist for 2'-FL/DFL, LNT, 6'-SL sodium salt, nor 3'-SL sodium salt. Nevertheless, the Codex Standards for 'Infant Formula and Formulas for Special

Medical Purposes Intended for Infants' (Codex Alimentarius, 2020) and for 'Follow-Up Formula' (Codex Alimentarius, 2017) contain provisions for 'optional ingredients' which are applicable to 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt.

J.2 Other National Standards and Regulations

The following national standards in jurisdictions with comparable regulatory processes are relevant to the current application:

- In the EU (and by association Switzerland), 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are authorised for use as novel food ingredients (EU, 2019, 2020, 2021a,b), for which provisions are laid down in the current consolidated version of the Union list of novel foods[4].
- In the U.S., Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation have been notified as GRAS to the U.S. FDA and have received no questions letters from the Agency (GRNs 815, 833, 880 and 881 – U.S. FDA, 2019a,b, 2020a,b), for which specifications are laid out in the GRAS notices (Glycom A/S, 2018a,b, 2019a,b).
- In Singapore, 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt have been authorised for use by the Singapore Food Agency (SFA). Permitted conditions of use of 2'-FL/DFL and LNT in infant formula were gazetted in the most recent amendment of the Food Regulations (SSO, 2021), while 6'-SL and 3'-SL sodium salts are currently laid down in approval letters from the SFA (Appendix X – **Commercial Confidential Information**) and will be included in the in the next planned amendment of the Food Regulations.
- In Israel, Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt have been authorised for use as novel foods by the Israel Ministry of Health (MOH), for which provisions are laid down in New Food Directives from the National Food Service Guidelines List (Israel MOH, 2022 a,b,c,d).
- In Brazil, Glycom's LNT, 6'-SL sodium salt, and 3'-SL sodium salt have been authorised for use by the Brazilian Health Regulatory Agency (ANVISA), for which provisions are provided in approval letters from ANVISA (Appendix X – **Commercial Confidential Information**).

K. Statutory Declaration

A signed Statutory Declaration is provided in Appendix I **(Confidential)**.

L. Checklists

Completed checklists relating to the information required for submission with this application are provided in Appendix II.

[4] Commission Implementing Regulation (EU) 2017/2470 of 20 December 2017 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. C/2017/8878. OJ L 351, 30.12.2017, p. 72–201. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?qid=1533914206967&uri=CELEX:32017R2470 (Latest consolidated version: 29/08/2022).

PART 3.3.3 – SUBSTANCES USED FOR A NUTRITIVE PURPOSE

A. Information on the Use of the Nutritive Substance

A.1 Purpose of the Use of the Substance

It is well established that human milk 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 milk. Considering that breastfeeding 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 many efforts over time to optimise the composition of infant formula to improve its suitability and to better reflect the composition of human milk (Carver, 2003; Thompkinson and Kharb, 2007; Stevens *et al.*, 2009; EFSA, 2014; Green Corkins and Shurley, 2016). Yet, even today with much progress and control over the composition of infant formula, human milk will always be the "gold standard" for infant nutrition.

Currently, the majority of infant formula and follow-on formula products on the market are formulated with mature cow's milk as a base. The biggest compositional difference between these infant formula products and human milk is that the latter contains a unique fraction of structurally diverse, non-digestible oligosaccharides which are not present in mature cow's milk to any significant degree.

In 2014, the European Food Safety Authority (EFSA) Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) provided its most recent updated Scientific Opinion on the essential composition of infant and follow-on formula (EFSA, 2014). The Opinion still represents one of the most up-to-date internationally accepted reviews of the science around infant feeding. Within this review, Section 5.4.2.2, Non-digestible (non-glycaemic) carbohydrates in human milk, states:

> *"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-chained 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). Fermentation products, i.e. SCFAs, contribute to the energy content of the diet, but to a lesser extent than glycaemic carbohydrates. Human milk oligosaccharides are not considered in the estimation of the energy content of the milk."*

Therefore, the addition of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt to infant formula products will result in the development of human milk substitutes that continue to better reflect the compositional profile of oligosaccharides of human 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 Alimentarius, 2020), where in Article 3.2.1 for optional ingredients it is stated that:

> *"… other 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."*

The addition of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt to infant formula products is therefore supported on a teleological basis and is consistent with efforts to produce infant formula products that more closely match the nutrient composition of human milk.

FSANZ previously recognised the beneficial effect of the presence of *Bifidobacterium* and *Lactobacillus* in the intestinal microflora in their evaluation of Proposal P306 (Addition of Inulin / FOS & GOS to Food – FSANZ, 2008) and Application A1055 (Short-chain Fructo-oligosaccharides – FSANZ, 2013a). In its initial Approval report for

Application A1155 (2'-FL and LNnT in infant formula and other products), FSANZ (2019d) concluded that 2'-FL and LNnT are likely to have a bifidogenic effect, defined by FSANZ as a proliferation and increase in the relative abundance of *Bifidobacterium* in the intestinal microflora. Evidence supporting this beneficial effect included *in vitro* bacterial growth and utilisation studies demonstrating that bifidobacteria have the ability to metabolise 2'-FL and LNnT (Asakuma *et al*., 2011; Ruiz-Moyano *et al*., 2013; Yu *et al*., 2013a,b; Garrido *et al*., 2015, 2016; Bunesova *et al*., 2016). The bifidogenic effect of 2'-FL and LNnT was further supported by results of a randomised clinical trial in breastfed infants (reference group) and formula-fed infants provided an infant formula supplemented with or without 2'-FL (up to 1.2 g/L) in combination with LNnT (up to 0.6 g/L) (Puccio *et al*., 2017; Berger *et al*., 2020). The microbial diversity (including a higher relative abundance of *Bifidobacterium*) and the metabolite profile in stools of infants supplemented with 2'-FL and LNnT approached that of breastfed infants compared to the infant formula group that did not receive HiMOs. Furthermore, results from a randomised clinical trial in adults provided 2'-FL, LNnT, or a 2:1 mixture of both HiMOs, each at doses of 5, 10, or 20 g/day for a duration of 14 days, suggest that the effect is not limited to infants and is likely dose dependent (Elison *et al*., 2016).

In the subsequent review report for Application A1155 conducted at the request of the Australia and New Zealand Ministerial Forum (FSANZ, 2020), FSANZ again noted that:

> *"A credible mechanism by which HMOs influence the composition of the gastrointestinal microbiome has been established through a number of ex vivo, in vitro and in silico studies on the utilisation of specific HMOs by bifidobacteria isolated from infant gastrointestinal tracts."*

Although human intervention and observational studies were considered supportive evidence of the bifidogenic effect of 2'-FL and LNnT, FSANZ (2020) noted that there was limited confidence in the size of the effect. The Independent Expert Advisory Group (IEAG), providing a third party assessment, agreed that there is a bifidogenic effect but that there is limited evidence from human studies to estimate the effect size. Still the IEAG also noted that:

> *"There are many different factors which influence infant health, and that it is not possible to determine a linear effect from the presence of one substance in human milk and a specific health outcome."*

FSANZ (2019d) also concluded from their assessment that 2'-FL is likely to have a pathogen binding effect against invasive *Campylobacter jejuni* (*C. jejuni*) strains through the establishment of a mechanism of action from cell culture (Ruiz-Palacios *et al*., 2003) and *in vivo* (Yu *et al*., 2016) studies demonstrating the binding of 2'-FL to the pathogen preventing attachment to the intestinal epithelial cells and infection. The IEAG reaffirmed this conclusion in their independent assessment (FSANZ, 2020). However, there was insufficient evidence to support beneficial effects of 2'-FL and LNnT on immune modulation or improved intestinal barrier function.

Beneficial-related effects of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt include microbiota-modulating effects, protective effect against pathogens, maintenance of intestinal barrier integrity and roles in immunomodulation. Supporting evidence for each of these effects is provided in the next section.

A.2 Supporting Evidence

A.2.1 Biological Role of HMOs

A.2.1.1 Microbiota-Modulating Effects

Historically, the discovery of HMOs was directly connected to their bifidogenic effect, since they were identified as the predominant "*bifidus factor*" of human milk (György, 1953; Gauhe *et al.*, 1954; György *et al.*, 1954a,b; György and Rose, 1955). Bifidobacteria are among the first microbes to colonise the infant gut (Bertelsen *et al*., 2016; Fukuda *et al.*, 2011), and are abundant in the gut microbiota of breastfed infants (Bezirtzoglou *et al.*, 2011; Yatsunenko *et al.*, 2012). Disruptions in early life microbiota composition has been hypothesised to contribute to the pathogenesis of defects in physiological, metabolic, immune, and neurological development (reviewed in Blanton *et al.*, 2016; Charbonneau *et al.*, 2016).

The association between HMO consumption and the gut microbiome composition has been widely reported *via* mechanistic studies and human observational studies (more recently reviewed in Thomson *et al.*, 2018; Zúñiga *et al.*, 2018; Sakanaka *et al.*, 2020; Zhang *et al*., 2021). Generally, the gut microbiome of exclusively formula-fed infants is characterised by a microflora profile that more closely resembles the digestive tract of adults; in contrast,

the microbiome of infants supplemented with HMOs are enriched with bifidobacteria and generally resemble that of breastfed infants.

As indicated in Section C.1 of this part of the application, there is limited digestion of HMOs in the upper gastrointestinal tract, resulting in the majority of ingested HMOs reaching the colon where they are fermented by intestinal microbiota. Since their discovery, HMOs have been demonstrated to support the growth of *Bifidobacterium*, with specific strains (*B. breve, B. bifidum, B. longum, and B. infantis*) harbouring genes for HMO-degrading enzymes (*e.g.*, glycosyl hydrolases, fucosidases, and sialidases), transporters, and carbohydrate-binding proteins (reviewed by Zhang *et al*., 2021). Apart from bifidobacteria, HMOs are utilised by other gastrointestinal bacteria (including *Bacteroides* and *Lactobacilli*), and metabolites from HMO digestion can also interact with gut microbes, all of which influence the overall composition and activity of the intestinal microbiota (reviewed by Zhang *et al*., 2021). A summary of the evidence supporting the bifidogenic effect of 2'-FL/DFL, LNT, 6'-SL, and 3'-SL is provided in the sections that follow, while tabular summaries of individual studies are provided in Annex II.

2'-FL/DFL

The bifidogenic effect of 2'-FL, the primary component of 2'-FL/DFL, has previously been recognised by FSANZ. Recent studies adding to the evidence base of 2'-FL's bifidogenic effect are tabulated in Annex II.

Fucosylated HMOs (including 2'-FL, DFL, and 3-FL) have been demonstrated in *in vitro* studies to significantly induce α-L-fucosidase activity (Yu *et al*., 2013a), support the selective growth of beneficial *Bifidobacteria spp*. and *Bacteroides spp*. (Asakuma *et al*., 2011; Yu *et al*., 2013a,b; Zabel *et al*., 2020; Salli *et al*., 2021) but not potentially pathogenic bacteria (Yu *et al*., 2013a,b; Salli *et al*., 2021), and reduce culture pH through the production of lactate and SCFAs (Yu *et al*., 2013a,b).

Greater than 40% DFL was consumed by almost all *Bifidobacteria* spp. type strain cultures (except *B. longum* ATCC15708) as well as *B. vulgatus* ATCC848 and *B. fragilis* ATCC25285 cultures supplemented with HMOs as the sole sugar source, from which most DFL-consuming bacteria exhibited significant induction of α-L-fucosidase activity (Yu *et al*., 2013a). In infant faecal microbiota cultures prepared from faecal samples collected from 9 healthy infants, representing comprehensive microbial communities, greater than 90% of 2'-FL and DFL were consumed from the HMO supplement (Yu *et al*., 2013b). Type strains able to utilize DFL generally produced lactate and/or SCFAs and reduced the pH in the culture medium (Yu et al., 2013a,b). Notably, across all 25 bacteria tested, Yu *et al*. (2013a) reported significant correlations between changes in fucosidase (DFL utilisation) and growth ($r = 0.642$, $P = 0.001$), changes in fucosidase and pH ($r = 0.592$, $P = 0.002$), and changes in organic acid production and pH ($r = 0.749$, $P < 0.001$).

Yu *et al*. (2013b) also noted a greater prebiotic effect of the HMO supplement primarily composed of fucosylated HMOs compared to frutco-oligosaccharides (FOS, positive control) fed at equal concentrations. Notably, infant faecal microbiota culture pH was significantly more reduced following incubation with the HMO supplement compared to FOS. Furthermore, the HMO supplement stimulated significantly greater growth than FOS of the two representative *B. longum* subsp. *infantis* type strains (ATCC15697 and JCM7007) evaluated.

Bacteria unable to utilise/grow on DFL are similar to those for 2'-FL and 3-FL, some of which are potentially pathogenic bacteria (*Clostridium* spp., *Lactobacillus* ATCC53103, *Enterococcus* ATCC29200, *Staphylococcus* spp., *Enterobacter* spp., *E. coli* K12, and *Clostridium perfringens* – Yu *et al*., 2013a,b). Likewise, relative gene frequencies of potentially pathogenic *Escherichia* spp. and *Clostridium perfringens* significantly declined in infant faecal microbiota cultures incubated with the HMO supplement compared to control (Yu *et al*., 2013b). While GOS has been reported to support the growth of potentially pathogenic bacteria in several *in vitro* studies (including *E. coli*, *Clostridium perfringens*, and *Cronobacter sakazakii* type strains) (Mao *et al*., 2014; Jakobsen *et al*., 2019; Salli *et al*., 2021), 2'-FL and DFL did not (Salli *et al*., 2021).

LNT

A number of mechanistic studies have demonstrated the ability of bifidobacteria to utilise LNT, including type strains for common species of *Bifidobacterium* present in the microbiota of breastfed infants such as *B. bifidum, B. longum* subsp. *infantis, B. longum* subsp. *longum,* and *B. breve* (LoCascio *et al.*, 2007; Asakuma *et al.*, 2011; Sela *et al.*, 2012; Ruiz-Moyano *et al*., 2013; James *et al.*, 2016; Özcan and Sela, 2018; Dogra *et al.*, 2021). Similarly, LNT was utilised by most bifidobacterial strains isolated from infant faeces during the first month of life (Matsuki *et al.*, 2016). A limited number of other bacteria have also been demonstrated to utilise LNT, including type strains of

Bacteroides thetaiotaomicron involved in cross-feeding of *Anaerostipes caccae* (Chia *et al*., 2020), as well as *Roseburia-hominis* and *Eubacterium ramulus* (Pichler *et al*., 2020).

Numerous studies have also characterised gene clusters and molecular pathways involved in the metabolism and transport of LNT by bifidobacteria (Suzuki *et al.*, 2008; Wada *et al.*, 2008a; Garrido *et al.*, 2011; Sela *et al.*, 2012; James *et al.*, 2016; Yamada *et al.*, 2017; Gotoh *et al*., 2018; James *et al*., 2018; O'Connell Motherway *et al.*, 2018; Özcan and Sela, 2018; Duar *et al*., 2020). Similar to LNnT, LNT is hydrolysed to galactose and lacto-*N*-triose II, but by a specific β-1,3-galactosidase rather than β-1,4-galactosidases. Alternatively, LNT is split in the middle into lacto-*N*-biose I (LNB) and lactose by endo-β-*N*-acetylglucosaminidases. These particular hydrolysing enzymes are rare in the bacterial domain but have been identified in *B. longum* subsp *infantis* and *B. bifidum* (Miwa *et al.*, 2010; Yoshida *et al.*, 2012; Sakanaka *et al.*, 2020).

6'-SL and 3'-SL

A number of mechanistic studies have demonstrated the ability of bifidobacteria to utilise sialyllactoses (including 6'-SL and 3'-SL) in culture experiments using type strains for common species of *Bifidobacterium* present in the microbiota of breastfed infants such as *B. bifidum*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*, and *B. breve* (Nakano *et al.*, 2001; Vester Boler *et al*., 2013; Yu *et al.,* 2013a; Bunesova *et al*., 2016; Moon *et al.*, 2016; Thongaram *et al.*, 2017; Cheng *et al.*, 2020; Chia *et al.*, 2021; Dogra *et al.*, 2021). Numerous studies have also demonstrated the inability of bacterial pathogenic type strains to grow on the sialyllactoses, including *Clostridium perfringens*, *Clostridioides difficile*, *Enterobacter aerogenes*, *Cronobacter sakazakii*, and *Escherichia coli* O1:K1:H7, to name a few (Yu *et al.,* 2013a; Hoeflinger *et al.*, 2015; Moon *et al.*, 2016).

Not all commensal bacterial species and type strains evaluated were able to utilise 6'-SL (Yu et al., 2013a; Bunesova *et al.*, 2016; Crost *et al.*, 2016; Cheng *et al*., 2020; Pichler *et al.*, 2020). 6'-SL and 3'-SL are hydrolysed by sialidases (also referred to as neuraminidases) which cleave the terminal sialic acid (reviewed in Juge *et al*., 2016 and Coker *et al*., 2021). Approximately 1,040 strains of human gut bacteria out of a reference collection of 2,662 bacterial genomes available from public sources (approximately 200 genera and 700 species) were predicted as sialic acid-utilizing strains (*i.e.*, identification of at least one gene encoding a sialidase, sialic acid transporter, or sialic acid catabolic enzyme), 40% of which were identified as possessing a sialidase (Coker *et al*., 2021). The presence of enzymes involved in the metabolism of HMOs in select species is the result of adaptive co-evolution of symbiotic commensals with the human host (Duranti *et al*., 2019). Indeed, there is evidence of the cross-feeding of substrates produced by bifidobacteria that utilise sialyllactoses, contributing to the growth of other important species that colonise the infant gut (Cheng *et al*., 2020; Chia *et al.*, 2020, 2021).

In two piglet feeding studies evaluating supplementation of sialyllactoses in the diet, there was no significant difference in the alpha-diversity of the faecal microbiome between dietary treatment groups (Jacobi *et al*., 2016; Monaco *et al.*, 2018). Significant microbiome differences were reported by Jacobi and colleagues in piglets fed 4 or 6 g/L 6'-SL or 3'-SL compared to control, while Monaco and colleagues reported minor effects on the relative abundance of specific microbes in piglets fed up to 760 mg/L sialyllactose. However, the relevance of results from microbiome analyses in piglet studies to effects in the infant microbiome should be interpreted with caution as piglets have little to no bifidobacteria (Loh *et al*., 2006; Pieper *et al.,* 2008; Petri *et al*., 2010), therefore.

A.2.1.2 Protective Effect Against Pathogens

The protective effect of HMOs against a range of pathogens are based on three mechanistic principles:

1) Inhibiting the growth and colonisation of pathogens by providing a competitive advantage to non-pathogenic commensals (*i.e.* the establishment of a more pathogen-resistant and lowering the pH of the gut as discussed in Section E.1.2);

2) Competitive binding to the carbohydrate recognition domains of pathogen-generated proteins/toxins and preventing pathogen adhesion;

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).

A summary of the evidence supporting the protective effect of 2'-FL/DFL, LNT, 6'-SL, and 3'-SL against pathogens is provided in the sections that follow, while tabular summaries of individual studies are provided in Annex II.

2'-FL/DFL

A pathogen-binding inhibitory effect of 2'-FL against *Campylobacter jejuni* has previously been recognised by FSANZ (FSANZ, 2019c). A summary of recent studies adding to the evidence base of 2'-FL's anti-infective effect against other pathogens is presented in Annex 2.

DFL has been demonstrated to reduce the growth of *Streptococcus agalactiae*, *Staphylococcus aureus*, and *Acinetobacter baumannii* (Craft *et al.*, 2018; Craft and Townsend, 2019). In a subsequent study, DFL was among the HMOs reported to have significant antimicrobial activity against Group B Streptococcus, with the highest average growth reduction over 24 hours (51 %) and the second highest average viability reduction (17 %) of the HMOs evaluated (Chambers and Townsend, 2020).

Several observational studies indicate an association between Secretor status and/or human milk HMO composition with the incidence of *Campylobacter*-associated diarrhoea (Morrow *et al*., 2004) and enterotoxigenic *E. coli*-associated diarrhoea (Newburg *et al.*, 2004a,b) in infants. A recent study evaluated the association between infant FUT2 Secretor status and the incidence of diarrhoea using logistic regression modelling. Genetic data and parent-reported incidence of diarrhoea were available for 4,971 infant-mother dyads from the Avon Longitudinal Study of Parents and Children (ALSPAC) cohort (Muthumuni *et al*., 2021). Overall, 24 % of infants were non-Secretors, and 27 % and 51 % of infants experienced diarrhoea from 0 to 6 and 6 to 18 months, respectively. Non-Secretor infants had 15% lower risk of diarrhoea before 6 months [odds ratio (OR): 0.85; 95 % confidence interval (CI): 0.72 to 0.99] and 30 % lower risk of diarrhoea from 6 to 18 months (OR: 0.70; 95 % CI: 0.61 to 0.81) compared to Secretors.

The association between mother Secretor status and the incidence of diarrhoea in infants was also evaluated in the same observational study (Muthumuni *et al*., 2021). Independent of the mother's Secretor status, breastfeeding for more than 6 months was strongly protective against diarrhoea in infants from 0 to 6 months (OR: 0.39; 95% CI: 0.33 to 0.45) and 6 to 18 months (OR: 0.81; 95% CI: 0.72 to 0.91). Following the addition of interaction terms for the mother's Secretor status to the model, the effect size was larger from 0 to 6 months for infants of non-Secretor mothers (67% reduced risk; OR: 0.33; 95% CI: 0.24 to 0.44) than Secretor mothers (59% reduced risk; OR: 0.41; 95% CI: 0.35 to 0.49), as well as from 6 to 18 months for infants of non-Secretor mothers (32% reduced risk; OR: 0.68; 95% CI: 0.53 to 0.86) than Secretor mothers (14% reduced risk; OR: 0.86; 95% CI: 0.75 to 0.99). However, there was no significant difference in the p-value for the interaction between breastfeeding and the mother's Secretor status for both age brackets (P for interaction= 0.18 for 0 to 6 months and 0.09 for 6 to 18 months). A major limitation of the study is that diarrhoea was parent-reported, and infant stool samples were not collected; as such, it was not possible to discriminate infectious diarrhoea caused by enteric pathogens.

LNT

Group B *Streptococcus* (GBS) can be passed from mothers to infants during childbirth and cause infection in new-borns (Department of Health, 2020). Unlike GOS, pooled HMOs and in particular neutral HMOs (including LNT) were demonstrated to significantly inhibit the growth of the three most common GBS serotypes III (strain COH1), Ia (strain A909) and V (strain NCTC10/84) in *in vitro* growth studies (Lin *et al.*, 2017). In subsequent studies, growth reduction of GBS was generally more effective by pooled HMOs compared to single HMOs, and antimicrobial activity tended to be strain specific (Craft *et al.*, 2018; Craft and Townsend, 2019; Chambers and Townsend, 2020).

Preliminary data from *in vitro* cell assays demonstrate the prevention of bacterial cellular adhesion by LNT and LNnT. Free oligosaccharides purified from the low-molecular-weight fraction of human milk (including neolacto- and lactotetraose as well as their fucosylated derivatives) and synthetic oligosaccharides inhibited the adhesion of *Streptococcus pneumoniae* to human pharyngeal cells (Andersson *et al.*, 1986). Similarly, the lacto- and neolactotetraoses fraction of human milk moderately inhibited the adhesion of enteropathogenic *E. coli* to Hep-2 cells (Cravioto *et al.*, 1991).

LNT and LNnT have also been demonstrated to bind the "VP8*" glycan binding domain of the VP4 spike protein of several rotavirus strains (Yu et al., 2014; Ramani et al., 2018; Sun *et al.*, 2018), though preliminary data suggest that these HMOs may not be sufficient to act as decoy receptors against P[11] rotaviruses (Ramani *et al.*, 2018).

While pooled HMOs prevented the attachment of *Entamoeba histolytica* to intestinal HT-29 cells by more than 80%, cytotoxicity induced by the protozoan parasite, whose major virulence factor is a lectin that binds galactose, was prevented in a dose-dependent manner by up to 80% by LNT alone (containing a residual galactose) but not

by HMOs with fucose residues linked to galactose at their terminal unit (such as 2'-FL and LNFP1) (Jantscher-Krenn *et al*., 2012).

6'-SL and 3'-SL

6'-SL and 3'-SL have been demonstrated to bind various viral strains in binding assays (Weis *et al.*, 1988; Kubota *et al.*, 2016, 2019; Wegener *et al.*, 2017). Both sialylactoses substantially reduced infectivity of rotavirus strains in African green monkey kidney epithelial cells (Vero cells) (Laucirica *et al.*, 2017), while only 3'-SL inhibited mumps virus entry (Kubota *et al.*, 2019). In haemagglutination inhibition assays, 6'-SL exhibited antiviral activity against select avian influenza subtype strains, while 3'-SL demonstrated antiviral activity against all subtype strains evaluated (Pandey *et al.*, 2018). In the same publication, 3'-SL reduced H9N2 infectivity in Madin Darby Canine Kidney Cells at lower than concentrations than 6'-SL, prompting 3'-SL to be evaluated further in an *in vivo* H9N2 infectivity chicken model. Viral loads from oral and cloacal swabs were lower but not significantly different in 3'-SL treated chickens challenged with a higher dose of H9N2 (8 HAU) compared to those not treated with 3'-SL, whereas no virus was detected in 3'-SL treated chickens challenged with a lower dose of H9N2 (0.8 HAU) while still being detected in non-3'-SL treated chickens.

In *in vitro* studies using Caco-2 cells, 6'-SL and 3'-SL have been reported to have an anti-adhesive effect against bacterial pathogen strains associated with diarrhoea (enteropathogenic *E. coli* serotypes O127:H6 and O119, *Salmonella enterica* subspecies *enterica* serotype *Fyris*, and *Vibrio cholerae* ATCC 14034) (Angeloni *et al*., 2005; Coppa *et al*., 2006). In a subsequent study evaluating the anti-adhesive effect of 6'-SL but not 3'-SL, adhesion *of E. coli* serotype O119 to Caco-2 cells was significantly inhibited by 6'-SL at a concentration occurring in human milk (0.6 g/L) compared to control without 6'-SL (Facinelli *et al.*, 2019).

A.2.1.3 Maintenance of Intestinal Barrier Integrity

The intestinal barrier function plays an important role in maintaining host health. The gastrointestinal immune barrier is not fully developed at birth, and intestinal barrier dysfunction is implicated in infant diseases such as necrotising enterocolitis (NEC) and neonatal short bowel syndrome (Miller and Burjonrappa, 2013; Halpern and Denning, 2015). While the aetiology and pathogenesis of NEC has not been fully elucidated, breastfed infants exhibit up to 10-fold lower risk of developing NEC than formula-fed infants (reviewed in Bode, 2018). Several studies have shown that human milk decreases the intestinal permeability and therefore enhances the physical barrier of an infant intestine (Halpern and Denning, 2015). A recent study reported that human colostrum but not commercial infant formula applied apically to paediatric enteroid monolayers enhanced epithelial barrier function by reducing ion permeability, stimulating epithelial cell differentiation, and enhancing tight junction function (Noel *et al*., 2021).

The protective effect of HMOs on gut barrier function has been examined using both *in vitro* and animal models (reviewed in Cheng *et al.*, 2021a). The ability of HMOs to support the intestinal barrier function can be explained through both direct and indirect mechanisms. Principally, HMOs are able to bind to G protein-coupled receptors expressed in the gastrointestinal tract. Direct effects on cells in the gastrointestinal tract have also been demonstrated in cell culture studies in which specific HMOs, individually and combined, had effects on cell cycle (including proliferation, differentiation, and apoptosis) and epithelial barrier function (translocation and transepithelial resistance). Indirectly, HMOs are thought to promote gut barrier function by modulation of the microbiota composition, formation of SCFA, and enhancement of the mucosal immune response. It has been shown that stimulating the growth of infant-dominate bifidobacteria by HMOs can enhance tight junction protein expression and immunomodulatory IL-10 in CaCo2 cells and intestinal epithelial cells, an effect that could not be observed when the bacteria grew on lactose (Chichlowski *et al*., 2012). In addition to the mechanistic studies, more recent work has examined the effect of HMOs in *in vivo* models (including murine models of necrotizing enterocolitis) and their influence on cell signalling and functional endpoints such as gut barrier integrity.

A summary of the evidence supporting improvement of intestinal barrier function by 2'-FL/DFL, LNT, 6'-SL, and 3'-SL is provided in the sections that follow, while tabular summaries of individual studies are provided in Annex II.

2'-FL/DFL

In an *in vitro* model of the crypt-villus axis, 2'-FL (along with LNnT and 6'-SL) dose-dependently inhibited cell proliferation in undifferentiated HT-29 and Caco-2Bbe intestinal cell cultures (Holscher et al., 2014). However, only

2'-FL at concentrations similar to human milk significantly increased markers of intestinal epithelial cell differentiation, including alkaline phosphatase activity in undifferentiated HT-29 cells and sucrase activity in well-differentiated Caco-2Bbe cultures. In a subsequent study, 2'-FL, 3'-SL, and 6'-SL individually and in combination inhibited proliferation of pre-confluent epithelial cells and promoted intestinal epithelial differentiation in the same crypt-villus axis model (Holscher *et al*., 2017). In a mouse model of intestinal adaptation following extensive ileocecal resection, 2'-FL administration changed the intestinal microbial community and resulted in an augmentation of adaptive responses including increased absorptive surface area, increased crypt depth, and increased villus height (Mezoff *et al*., 2016).

In an *in vitro* study evaluating the effect of HMOs (individually and in combination) on epithelial barrier function, 2'-FL alone (and to a lesser extent LNT) dose-dependently decreased fluorescein isothiocyanate–dextran KDa (FD4) translocation following inflammatory challenge, an effect that was reduced in blends not containing 2'-FL (Natividad *et al*., 2020). These results were corroborated in an *in vivo* study conducted in young Sprague Dawley rats fed 2'-FL fortified diets (alone or in combination with 3'-SL) at a level providing a similar average dose as breastfed infants for 8 weeks, where it was reported that intestinal permeability was reduced in females fed the HMO-fortified diets who had significantly lower plasma FD4 compared to controls (Chleilat *et al.*, 2020). In male mice fed low- and high-fat diets, 2'-FL supplementation for 8 weeks significantly decreased paracellular and transcellular permeability of the cecum compared to controls, as evidenced by significant decreases in FD4 and horseradish peroxidase Type IV (HRP4) flux *ex vivo* following necropsy (Lee *et al*., 2021).

The glycocalyx of gut epithelial cells plays an important role in preventing pathogen entry into the cell. Fucosylated HMOs including 2'-FL were demonstrated *in vitro* to stimulate the development of the glycocalyx of Caco2 epithelial cells, such as thickness and area of coverage. (Kong *et al*., 2019). 2'-FL also significantly induced essential genes for mucus barrier function in human goblet cells and prevented barrier disruption in human T84 cells (Figueroa-Lozano *et al*., 2021). Furthermore, fermentation products of 2'-FL incubated alone or in combination with LNnT significantly reduced paracellular permeability in Caco2 cell monolayers and increased gene expression of the claudin-8 membrane protein involved in the development of tight junctions, again demonstrating the indirect influence of HMO-derived metabolites on intestinal barrier function (Šuligoj *et al.*, 2020).

LNT

In receptor assays, LNT activated the expression of the G-protein coupled receptor GPR35 (expressed in the gastrointestinal tract, including intestinal surface epithelium), and induced an even greater response when combined with 6'-SL in a 1:1 ratio at the same concentration (Foata *et al*., 2020).

LNT's constitutional isomer LNnT has been demonstrated to impact growth characteristics of human, non-transformed epithelial cells *in vitro* at physiologically relevant concentrations by significantly decreasing cell proliferation, together with significantly increased cells in G2/M and S phases and significantly decreased cells in G0/G1 (Hester and Donovan, 2012). In a subsequent study, LNnT also dose-dependently inhibited cell proliferation in undifferentiated HT-29 and Caco-2Bbe intestinal cell cultures in an *in vitro* model of the crypt-villus axis (Holscher *et al*., 2014).

6'-SL and 3'-SL

In a preliminary *in vitro* study, a sialyllactose mixture (containing 40% 6'-SL, 10% 3'-SL, and 50% sialic acid) significantly decreased the proliferation of human, non-transformed epithelial cells, where, as observed for LNnT, the sialyllactose mixture significantly increased cells in G2/M and S phases and significantly decreased cells in G0/G1 (Hester and Donovan, 2012). Similar to 2'-FL and LNnT, 6'-SL also dose-dependently inhibited cell proliferation in undifferentiated HT-29 and Caco-2Bbe intestinal cell cultures in an *in vitro* model of the crypt-villus axis (Holscher *et al*., 2014). In a subsequent study, 6'-SL, 3'-SL, and 2'-FL individually and in combination inhibited proliferation of pre-confluent epithelial cells and promoted intestinal epithelial differentiation in the same crypt-villus axis model (Holscher *et al*., 2017).

6'-SL was also reported to activate GPR35 expression in the same receptor assays as those for LNT, where the response was enhanced when both HMOs were administered in combination (Foata *et al*., 2020).

In vivo, female Sprague Dawley rat pups fed diets fortified with 3'-SL (alone or in combination with 2'-FL) at a level providing a similar average dose as breastfed infants for a duration of 8 weeks were observed to have significant reductions in intestinal permeability (assessed according to plasma FD4) compared to controls (Chleilat *et al*., 2020).

A.2.1.4 Roles in Immunomodulation

Studies have shown a relationship between exclusive breastfeeding and the mitigation of immune system-implicated diseases including asthma, allergies, inflammatory bowel disease, Type 1 diabetes, celiac disease, and leukemia (reviewed in Kulinich and Liu, 2016; Ayechu-Muruzabal *et al.*, 2018; Triantis *et al.*, 2018; Pretorius *et al.*, 2018; Doherty *et al.*, 2018; Wiciński *et al.*, 2020).

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 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, DFL, LNnT or 6'-SL can interact with immune cells affecting immune cell activation and cytokine production (Velupillai and Harn, 1994; Zhu *et al.*, 2003, 2005; Amin *et al.*, 2008; Rabquer *et al.*, 2012; Comstock *et al.*, 2014; Newburg *et al.*, 2016; Zehra *et al*., 2018; Cheng et al., 2021b). Furthermore, sialylated HMOs have been shown to interact with siglecs (lectins implicated in immune function), selectins (implicated in leucocyte adhesion), integrins, and have influence on leukocyte interactions with endothelial and platelet cells as shown through *in vitro* investigations (reviewed in Triantis *et al.*, 2018).

In animal studies, the HMOs have been shown to mitigate inflammatory responses in models of NEC (2'-FL and 6'-SL: Sodhi *et al*., 2021), arthritis (3'-SL: Jeon *et al*., 2018; Kang *et al*., 2018), dermatitis (3'-SL: Kang *et al*., 2020), psoriasis (2'-FL: Lei *et al.*, 2020), colitis (2'-FL: Li *et al.*, 2020), food allergy (2'-FL and 6'-SL: Castillo-Courtade *et al*., 2015), intracerebral haemorrhage (2'-FL: Hung *et al*., 2021), and stress-induced inflammation (sialyllactoses: Allen *et al*., 2019). Contrary to these findings, 3'-SL supplementation had a pro-inflammatory effect in a mouse model of colitis induced by IL-10 deficiency[5] by stimulating mesenteric lymph node CD11c+ dendritic cells associated with mucosal innate immunity through Toll-like receptor 4 (TLR4) signalling, leading to the induction of cytokine production (Kurakevich *et al.,* 2013).

A.2.2 Randomised Controlled Infant Clinical Study

The results of benefit-related outcomes from a randomised controlled clinical trial in infants receiving infant formula supplemented with 1.5 or 2.5 g/L of a five-HMO blend containing Glycom's 2'-FL/DFL, LNT, 6'-SL and 3'-SL HiMOs through to 6 months of age have been published (Bosheva *et al*., 2022). The results support the physiological functions of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt described in the previous section, namely microbiota-modulating effects and protective effect against pathogens (by analysis of faecal microbiome, pH, and organic acids), as well as maintenance of intestinal barrier integrity and roles in immunomodulation (by analysis of faecal biomarkers).

Safety-related outcomes of this infant clinical study are discussed in Section C.2.2 of this part of the application (Cohen, 2022 [unpublished]).

Double-Blind, Randomised, Controlled, Gut Maturation Study of Formula for Infants Containing Five-HMO Blend (Bosheva et al., 2022)

The gut maturation effects of a standard bovine milk-based whey predominant term infant formula containing a five-HMO blend was investigated in a double-blind, randomised, controlled, multicentre trial[6] (Bosheva *et al*., 2022; Clinical Trial Registry NCT03722550). Healthy term infants 7 to 21 days of age at enrolment and whose parents had elected to formula feeding prior to enrolment were eligible for the study. Enrolled infants were randomised to the control group (CG) fed a standard infant formula with no HiMOs, test group 1 (TG1) fed the same standard infant formula with 1.5 g/L of the five-HMO blend, or test group 2 (TG2) fed the standard infant formula with 2.5 g/L of the five-HMO blend. A non-randomised human milk-fed group (HMG) was enrolled in parallel as a reference. Participants consumed the assigned infant formula (or breastmilk in HMG) from enrolment to 6 months of age, with the introduction of complementary foods at 4 months of age.

The five-HMO blend was composed of 2'-FL, DFL, LNT, 3'-SL, and 6'-SL. Concentrations of the individual HiMOs in the standard infant formula fed to TG1 and TG2, which are within the range of concentrations reported to naturally occur in human milk, are provided in Table A.2.2-1 below.

[5] Mice were also deficient in the enzyme responsible for 3'-SL biosynthesis (*Il10*$^{-/-}$; *St3gal4*$^{-/-}$).

[6] The trial was conducted at 32 study sites in Bulgaria, Hungary, and Poland.

Table A.2.2-1 Concentrations of Individual HiMOs from the Five-HMO Blend in Test Formulas

HiMO	TG1 (1.5 g/L total HMOs)	TG2 (2.5 g/L total HMOs)
2'-Fucosyllactose (g/L)	0.87	1.45
Difucosyllactose (g/L)	0.10	0.14
Lacto-*N*-tetraose (g/L)	0.29	0.48
6'-Sialyllactose (g/L)	0.14	0.24
3'-Sialyllactose (g/L)	0.11	0.18

HiMO = human-identical milk oligosaccharide.

Faecal samples were collected at enrolment, 3 months of age, and 6 months of age for microbiome, pH organic acids, and biomarker analyses. Microbial DNA was extracted by shotgun sequencing. The relative abundance of bacterial taxonomy was calculated using the metagenomic species (MGS) approach, and a phylogenetic tree connecting the MGSs was generated, from which alpha diversity (Faith's phylogenetic diversity index) and beta diversity (weighted UniFrac distance) were calculated. Richness and Shannon diversity at the level of gene, MGS and genus were also calculated as an index of alpha diversity. Faecal pH was assessed using pH indicator paper; organic acids (including lactate, acetate, butyrate, isobutyrate, propionate, valerate, and isovalerate) were assessed by a previously validated liquid chromatography-tandem mass spectrometry method; and biomarkers were assessed using commercially available enzyme-linked immunosorbent assays.

Of the 693 randomised formula-fed infants and 96 non-randomised human milk-fed infants, stool samples were analysed for 535 infants (CG: 155 infants; TG1: 158 infants; TG2: 153; HMG: 69 infants). Baseline characteristics were similar among all groups, with the exception of longer maternal and paternal education in years and slightly older gestational age at birth in weeks in HMG compared to formula-fed groups.

Alpha- and beta-diversity indicated a shift of the composition of gut microbiota in the five-HMO blend groups towards that of HMG. Of the alpha-diversity indexes, Shannon diversity (at the gene and genus levels) was significantly lower in TG1 than CG at 3 months of age, while Faith's phylogenetic diversity, richness, and Shannon diversity (at the gene, MGS, and genus levels) were significantly lower in TG1 than CG at 6 months of age. In TG2, richness and Shannon diversity (at the genus level) were significantly lower than CG at 6 months. TG1 and TG2 indexes were approaching that of HMG, which were all significantly lower than the formula-fed groups. Beta-diversity was significantly different in TG1 and TG2 compared to CG at both 3 and 6 months of age. There was a shift of the gut microbiota in infants receiving formulas with the five-HMO blend towards that of HMG (no significant difference between TG1 and HMG at 6 months[7]), while beta-diversity of CG remained significantly different and more distant from HMG. Furthermore, the microbiota composition of formula-fed infants receiving the five-HMO blend, regardless of delivery mode or stratified by delivery mode (*i.e.*, caesarean- or vaginally-delivered), shifted closer to that of vaginally delivered infants from HMG at 3 and 6 months of age.

Differences in the abundance of bifidobacteria between infants receiving formulas with the five-HMO blend and CG were reported, with TG1 and TG2 approaching HMG. At 6 months of age, the relative abundance of *Bifidobacterium* was higher by approximately 45% in TG1 and TG2 compared to CG but comparable to HMG. A similar pattern was observed when stratified according to delivery mode, particularly for caesarean-delivered infants. The relative abundance of infant-type *Bifidobacterium* species[8] was higher in TG1 than CG at 3 months, and both TG1 and TG2 than CG at 6 months, but comparable between TG1 and HMG by 6 months. Specifically, the relative abundance of *Bifidobacterium longum ssp. infantis* (*B. infantis*) was significantly higher in TG1 and TG2 than CG at 3 months, which persisted in TG1 compared to CG and tended to be higher in TG2 compared to CG at 6 months, approaching HMG in all cases. It should be noted, however, that B. *infantis* was also significantly higher in TG1 than CG as enrolment. Furthermore, the relative abundance of several other major bacteria taxa (Streptococcus, Lactobacillus, Clostridia, Peptostreptococcaceae) were significantly different in TG1 and/or TG2 from CG and approaching HMG at 3 and/or 6 months.

The relative abundance of toxigenic *Clostridioides* (former *Clostridium*) *difficile* (*C. difficile*) based on the presence of virulence genes was significantly lower (by 75 to 85%) in TG1 and TG2 than CG at 3 and 6 months of age. The prevalence of toxigenic *C. difficile* at 3 months tended to be lower in TG1 (6.5% of infants) and TG2 (5.7% of

[7] Although TG2 remained significantly different from HMG at 6 months ($p<0.04$), the level of significance decreased from 3 ($P<0.001$) to 6 months ($p=0.04$).

[8] Defined as including *Bifidobacterium longum* subsp. *infantis*, *Bifidobacterium longum* subsp. *longum*, *Bifidobacterium bifidum*, and *Bifidobacterium breve*.

infants) than CG (13.3% of infants). At 6 months, the proportion of infants with toxigenic *C. difficile* was significantly lower in TG1 (10.6% of infants) and TG2 (6.0% of infants) compared to CG (27.9% of infants) and comparable with HMG (10.0% of infants). Other pathogens of interest were not investigated further due to low prevalence[9].

Faecal secretory immunoglobulin A (sIgA), a marker of intestinal immune response, was significantly higher (by 43 to 53%) in the five-HMO blend groups than CG at 3 months of age and persisted in TG2 at 6 months of age. Faecal alpha-1-antitrypsin (AAT), a marker of intestinal permeability which is known to decrease during infancy, was significantly lower in the five-HMO blend groups compared to CG at 3 months. Faecal calprotectin, a marker of gut inflammation, trended lower in TG1 compared to CG at 3 months, reaching statistical significance at 6 months.

Mean faecal pH was significantly lower in the five-HMO blend groups than CG at 3 and 6 months of age. While mean faecal pH was comparable at 3 months between the five-HMO blend groups and HMG, this did not persist into 6 months at which point HMG showed the lowest pH. Significant differences were observed in the faecal organic acid profile between TG1 and/or TG2 and CG, often approaching levels in HMG. Specifically, the main end-products produced by bifidobacteria, acetate and lactate, involved in lowering the pH in the colon, were significantly higher in infants receiving the five-HMO blend than CG.

Taken together, the study investigators concluded that consumption of infant formula with the five-HMO blend through to 6 months of age "*supports the development of the intestinal immune system and gut barrier function and shifts the gut microbiome closer to that of breastfed infants with higher bifidobacteria, particularly B. infantis, and lower toxigenic Clostridioides difficile*."

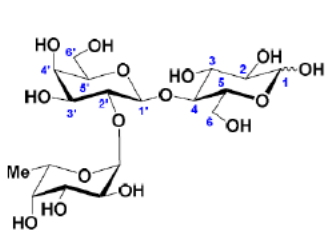
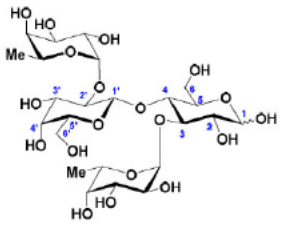
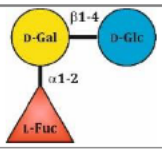
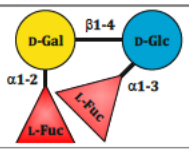
B. Technical Information on the Use of the Nutritive Substance

B.1 Identification

2'-FL, DFL, LNT, 6'-SL, and 3'-SL obtained from microbial fermentation are chemically and structurally identical to the same respective HMOs that are naturally present in human milk, as confirmed by ^{1}H- and 2D-nuclear magnetic resonance (NMR)-spectroscopy and mass spectrometry. Please see Appendix III **(Confidential Commercial Information)** for a comparison of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL obtained from fermentation to authentic samples isolated from human milk.

2'-FL and DFL belong to the "fucosylated" sub-fraction of HMOs, oligosaccharides that contain the sugar fucose. Structurally, 2'-FL is a trisaccharide derived from lactose by addition of a fucose sugar that is linked by an α-(1→2) bond, while DFL is a tetrasaccharide derived from 2'-FL by the addition of a second fucose sugar to the 3-glucose position of 2'-FL. A comparison of 2'-FL and DFL structures is provided in Table B.1-1.

Table B.1-1 Comparison of 2'-FL and DFL Structures

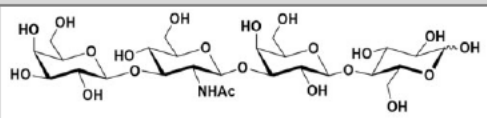
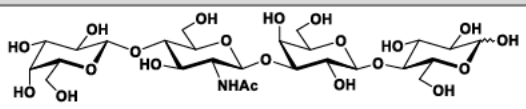
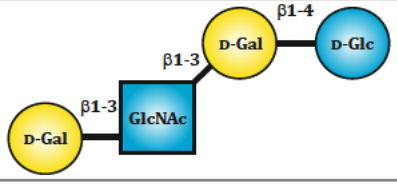
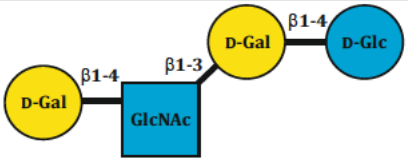
	2'-FL	DFL
Molecular Structure		
Symbol Nomenclature		
		

2'-FL = 2'-fucosyllactose; DFL = difucosyllactose.

[9] Including *Campylobacter jejuni*, *Campylobacter coli*, *Clostridium perfringens,* enteropathogenic *Escherichia coli* (EPEC), and enterotoxigenic *Escherichia coli* (ETEC), *Klebsiella pneumoniae* and *Salmonella enterica.*

LNT and its constitutional isomer LNnT belong to the "neutral core" sub-fraction of HMOs. Structurally, LNT and LNnT share a highly similar chemical structure as they are both tetrasaccharides derived from lactose by subsequent addition of *N*-acetylglucosamine (GlcNAc) and galactose. The notable difference between these HMOs is that LNT contains a terminal Gal-β(1-3)-GlcNAc linkage, while LNnT contains a terminal Gal-ß(1-4)-GlcNAc linkage. A comparison of LNT and LNnT structures is provided in Table B.1-2.

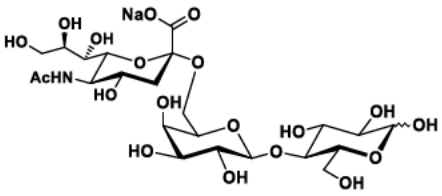
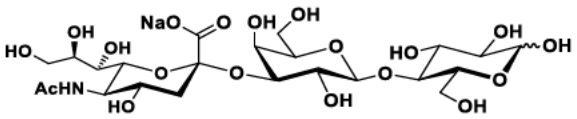
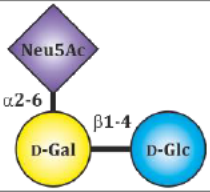
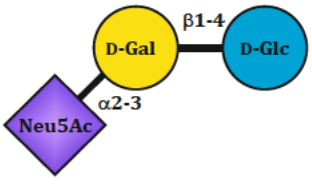
Table B.1-2 Comparison of LNT and LNnT Structures

	LNT	LNnT
Molecular Structure		
Symbol Nomenclature		

LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose.

6'-SL and its constitutional isomer 3'-SL belong to the acidic sub-fraction of HMOs, also called "sialylated HMOs". Structurally, 6'-SL and 3'-SL share a highly similar chemical structure as they are both trisaccharides derived from lactose by subsequent addition of sialic acid[10]. The notable difference between these HMOs is that 6'-SL contains sialic acid at the 6 position of the D-galactose unit, while 3'-SL sialic acid at the 3 position of the D-galactose unit. A comparison of 6'-SL and 3'-SL structures is provided in Table B.1-3.

Table B.1-3 Comparison of 6'-SL and 3'-SL Structures

	6'-SL	3'-SL
Molecular Structure		
Symbol Nomenclature		

2'-FL = 2'-fucosyllactose; DFL = difucosyllactose.

[10] Sialic acid is the more common (but colloquial) synonym for *N*-acetylneuraminic acid (abbreviated either as NANA or Neu5Ac).

B.2 Chemical and Physical Properties

B.2.1 Chemical and Physical Properties of 2’-FL/DFL, LNT, 6’-SL and 3’-SL

Glycom’s 2'-FL, DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt products are white to off-white amorphous powders or agglomerates. They are readily soluble in aqueous solutions (max. 400 to 500 mg/mL, 25 °C), with poor solubility in any organic solvents.

Chemical and physical properties of 2'-FL/DFL, LNT, 6'-SL, and 3'-SL obtained from microbial fermentation are provided in Table B.2.1-1 below.

Table B.2.1-1 Chemical and Physical Properties of 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3'-SL Sodium Salt

Trade Name:	GlyCare™ 2FL/DFL 8001		GlyCare™ LNT 8001	GlyCare™ 3SL 9001	GlyCare™ 6SL 9001
	2'-Fucosyllactose (2'-FL, 2'FL, 2FL, 2-FL)	**Difucosyllactose (DFL, LDFT)**	**Lacto-*N*-tetraose (LNT)**	**3'-Sialyllactose (3'-SL, 3'SL, 3SL, 3-SL) sodium salt**	**6'-Sialyllactose (6'-SL, 6'SL, 6SL, 6-SL) sodium salt**
Synonyms	2'-*O*-Fucosyllactose, 2'-*O*-L-Fucosyl-D-lactose, 2'-Fucosidolactose, H-2g (glucose analog of histo-blood group H antigen)	Lacto-difuco-tetraose (LDFT)	Not available	3'-*O*-(*N*-Acetylneuraminyl)-lactose sodium salt, 3'-O-Sialyllactose sodium salt, 3'-Lactaminyllactose sodium salt	6'-*O*-(*N*-Acetylneuraminyl)-lactose sodium salt, 6'-O-Sialyllactose sodium salt, 6'-Lactaminyllactose sodium salt
International Union of Pure and Applied Chemistry (IUPAC) Name:	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-D-glucose	α-L-Fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-D-glucose	β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucose	*N*-Acetyl-α-D-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt	*N*-Acetyl-α-D-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt
IUPAC Abbreviation (extended):	α-L-Fuc*p*-(1-2)-β-D-Gal*p*-(1-4)-D-Glc	α-L-Fuc*p*-(1-2)-β-D-Gal*p*-(1-4)-[α-L-Fuc*p*-(1-2)]-D-Glc	β-D-Gal*p*-(1-3)-β-D-GlcNAc*p*-(1-3)-β-D-Gal*p*-(1-4)-D-Glc	α-D-Neu*p*5Ac-(2-3)-β-D-Gal*p*-(1-4)-D-Glc, sodium salt	α-D-Neu*p*5Ac-(2-6)-β-D-Gal*p*-(1-4)-D-Glc, sodium salt
IUPAC Abbreviation (condensed):	Fuc-(α1-2)-Gal-(β1-4)-Glc	Fuc-(α1-2)-Gal-(β1-4)-[Fuc-(α1-3)]-Glc	Gal-(β1-3)-GlcNAc-(β1-3)-Gal-(β1-4)-Glc	Neu5Ac-(α2-3)-Gal-(β1-4)-Glc, sodium salt	Neu5Ac-(α2-6)-Gal-(β1-4)-Glc, sodium salt
Chemical Abstracts Service (CAS) Name:	*O*-6-Deoxy-α-L-galactopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)-D-glucose	*O*-6-Deoxy-α-L-galactopyranosyl-(1→3)-*O*-[6-deoxy-α-L-galactopyranosyl-(1→2)-*O*-β-D-galactopyranosyl-(1→4)]-D-glucose	*O*-β-D-Galactopyranosyl-(1→3)-*O*-2-(acetylamino)-2-deoxy-β-D-glucopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→4)-D-glucose	*O*-(*N*-acetyl-α-neuraminosyl)-(2→3)-*O*-*β*-D-galactopyranosyl-(1→4)-D-Glucose, sodium salt (1:1)	*O*-(*N*-acetyl-α-neuraminosyl)-(2→6)-*O*-*β*-D-galactopyranosyl-(1→4)-D-glucose, sodium salt (1:1)
CAS Registry Number:	41263-94-9	20768-11-0	14116-68-8	128596-80-5	157574-76-0
Chemical Formula:	$C_{18}H_{32}O_{15}$	$C_{24}H_{42}O_{19}$	$C_{26}H_{45}NO_{21}$	$C_{23}H_{38}NO_{19}Na$	$C_{23}H_{38}NO_{19}Na$
Molecular Mass (Weight):	488.44	634.58	707.63	655.53	655.53

B.2.2 Bulk Stability

Storage (real-time and accelerated) and stressed (forced) stability studies on the individual pure ("bulk") powdered 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt products produced by microbial fermentation, as described herein, have been conducted in accordance with International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guidelines (*Stability Testing of New Drug Substances and Products*) (ICH, 2003) in order to:

1. Test the stability of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt during storage;
2. Investigate degradation pathways when exposed to selected stress factors; and
3. Define the optimal storage conditions and corresponding re-test dates or shelf-lives.

The real-time 5-year stability study [25 °C, 60 % relative humidity (RH)] is currently ongoing with results available up to 48 months of storage. Chemical, physical, microbiological, and sensory testing indicate that the 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt products are stable when stored at ambient room temperature for at least 48 months.

Under accelerated conditions (40°C, 75% RH) for a period of 2 years, results from chemical, physical, microbiological, and sensory testing indicate that there are no changes in organoleptic properties, no appreciable degradation, no changes in impurity profile, and no alterations in the microbiological quality of the 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt products. Based on the results of the accelerated stability study and taking the Arrhenius equation into account (Peleg *et al*., 2012), the stability of the 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt can be calculated to be at least 5 years when protected from light and stored at room temperature and ambient humidity.

Analytical results of the real-time 5-year stability studies (25 °C, 60 % RH) (up to 48 months of storage) and the 2-year accelerated stability studies of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are provided in Appendix IV **(Confidential Commercial Information)**.

The stress and forced stability studies aimed to identify the likely degradation products under harsh, stress conditions. Conditions and results of the stress and forced stability studies for each HiMO product are described below.

2'-FL/DFL

Forced stability tests of the bulk 2'-FL/DFL in aqueous solutions were performed at 60 and 80°C for 8 and 4 weeks of storage, respectively. These solids can be characterised by the next powder properties and by the next pH values when 5% solutions are made from them:

- Amorphous 2'-FL/DFL powder at acidic pH (4.1 and 4.5)
- Crystalline 2'-FL powder at slightly acidic pH (5.4) as reference
- Amorphous 2'-FL/DFL powder at neutral pH (6.2 and 7.0)

The results of these studies showed the action of two potential pH-dependent chemical degradation pathways in aqueous solutions of 2'-FL/DFL, namely hydrolysis at pH <5.0 and isomerization at pH >6.0. At neutral pH, 2'-FL and DFL underwent minor isomerization to fucosyl-lactulose and difucosyl-lactulose, correspondingly. At low pH, 2'-FL hydrolysed mainly to fucose and lactose, and slightly to 2-fucosyl-galactose and glucose; the hydrolyzation of DFL to 3-fucosyllactose and fucose was not reported. The latter reaction has been documented in an additional forced stability test conducted on 2'-FL/DFL syrup, thermally treated (80°C for 1 hour) and stored at 60°C for 4 weeks.

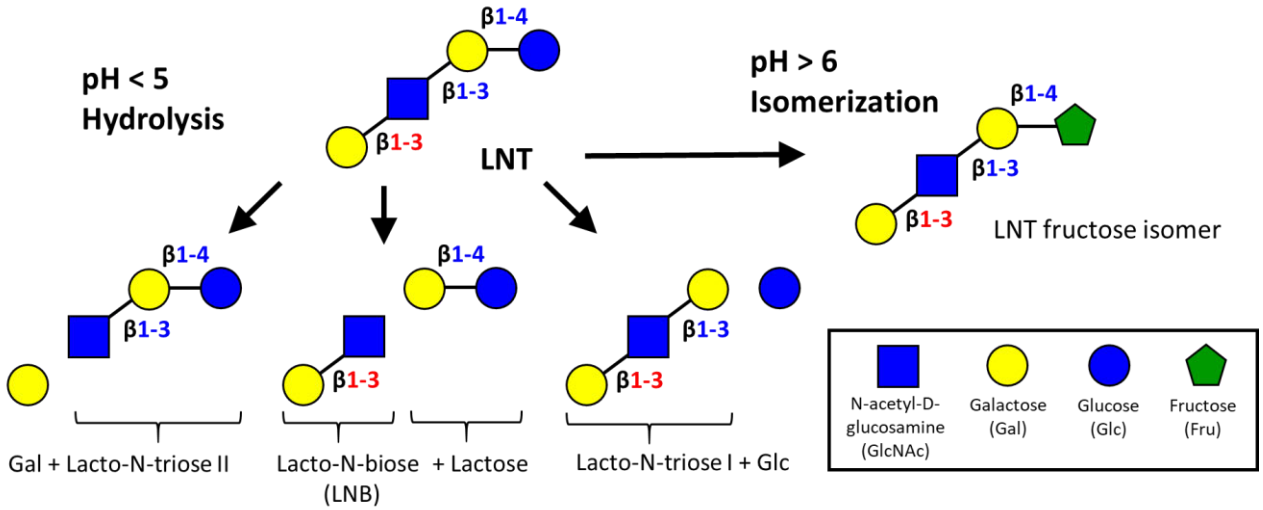
LNT

Forced stability tests of the solid bulk LNT product in the following aqueous solutions were performed at 60 and 80°C for 8 and 4 weeks of storage, respectively. These solids can be characterised by the next powder properties and by the next pH values when 5% solutions are made of them:

- Amorphous LNT powder with slightly acidic pH (4.5)
- Crystalline LNT powder with neutral pH (6.3) as reference
- Amorphous LNT powder with neutral pH (6.8)

The results of these studies showed two potential pH-dependent chemical degradation pathways in aqueous solutions of the LNT product, namely hydrolysis at pH < 5.0 and isomerisation at pH > 6.0. Theoretically, four predominant degradation pathways can be observed for LNT (Figure B.2.2-1), including three hydrolysis processes resulting in lacto-*N*-triose II and galactose, D-lactose and lacto-*N*-biose (LNB, Gal-GlcNAc), and D-glucose and lacto-*N*-triose I (Gal-GlcNAc-Gal) (Kuhn *et al*., 1956), as well as one transformation (isomerisation) reaction of LNT into the "LNT fructose isomer". None of the abovementioned degradation products are of safety concern as they are components of milk and milk products. The lacto-*N*-trioses and LNB are major building blocks of free oligosaccharides and glyco-conjugates present in human milk and gastrointestinal mucosa (Balogh *et al*., 2015; Bidart *et al.,* 2017), and constitute important growth factors for selected *Bifidobacterium* strains, which are predominant in the intestines of breast-fed infants (Wada *et al*., 2008b; Kiyohara *et al.,* 2009).

Figure B.2.2-1 Degradation Pathways of LNT in Aqueous Solutions



LNT = lacto-*N*-tetraose.

At neutral pH, the LNT product (both crystalline and amorphous form) undergoes minor isomerisation to the LNT fructose isomer. At slightly acidic pH, amorphous LNT was hydrolysed to D-glucose and lacto-*N*-triose II. It has been concluded that the optimal stability of LNT powder is observed at a pH between 5 and 6 (optimum close to 5.5), where neither the hydrolysis processes (occurring below pH 5) nor the isomerisation process (occurring above pH 6) is pronounced.

6'-SL and 3'-SL

Forced, thermal stability tests of the bulk 6'-SL and 3'-SL sodium salt ingredients in powdered solid state were performed at 80°C for 28 days of storage at two different humidity conditions: ambient and high humidity.

For both 6'-SL and 3'-SL sodium salts, there was negligible concurrent increase of lactose and sialic acid during the storage period. Slight isomerization of 6'-SL sodium salt to 6'-sialyl-lactulose and 3'-SL sodium salt to 3'-sialyl-lactulose were observed, both of which was more pronounced in an increased humidity condition.

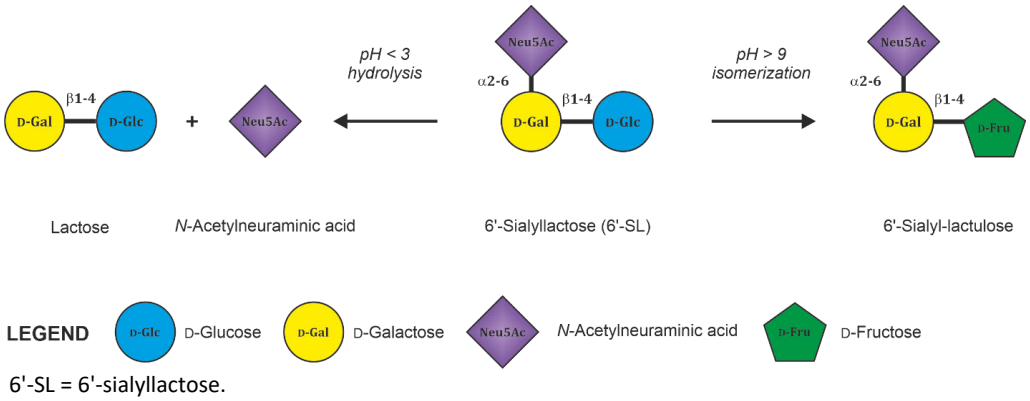
In aqueous solutions, forced stability tests of the bulk 6'-SL and 3'-SL sodium salt ingredients were conducted under the following stress conditions:

- Wide pH range (from 3.0 to 9.0) at 35°C over a period of 28 days.
- Acid (0.1 N HCl) and base (0.01 N NaOH) at 35°C over a period of 24 hours.
- Oxidation agents: 0.1% H_2O_2 at 35°C and 50% 4,4'-azobis(4-cyanovaleric acid) (ACVA) at 25°C over a period of 24 hours.

For 6'-SL sodium salt, there were two potential pH-dependent chemical pathways in the aqueous solutions, namely hydrolysis at pH < 3.0 and isomerization at pH > 9.0 (Figure B.2.2-2). In aqueous solution, 6'-SL was stable at neutral pH (6.7 and 6.9) at 35°C for 1 month, whereas, at slightly acidic solution (pH=5.0) at 35°C for 1 month only minor (3%) hydrolysis of 6'-SL to sialic acid and lactose was observed. However, under acidic conditions (at pH 3.0, 35°C for 1 month or at 0.1 N HCl, 35°C for 24 hours), 6'-SL almost completely hydrolysed

to sialic acid and lactose. While, under basic conditions (at pH 9.0, 35°C for 1 month or at 0.01 N NaOH 35°C for 24 hours) significant (10 to 30%) isomerization to 6'-sialyl-lactulose was noticed.

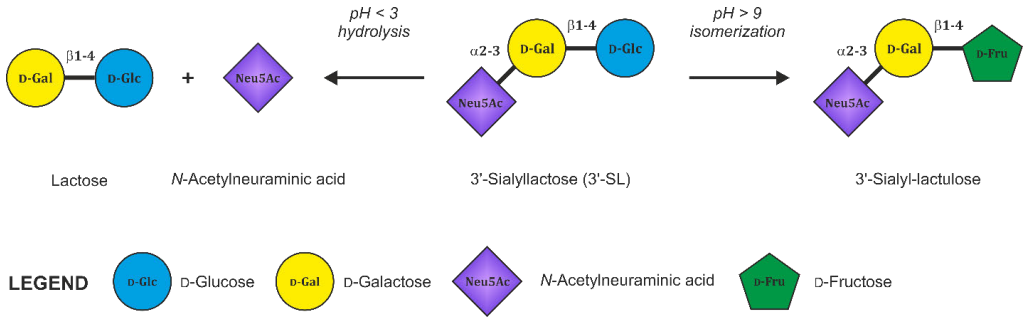
Figure B.2.2-2 Degradation Pathways of 6'-SL in Aqueous Solutions



6'-SL = 6'-sialyllactose.

For 3'-SL sodium salt, there were two potential pH-dependent chemical pathways in the aqueous solutions, namely hydrolysis at pH < 3.0 and isomerization at pH > 9.0 (Figure B.2.2-3). The data from this study revealed that 3'-SL in aqueous solution was stable at neutral pH (6.9) at 35°C for 1 month. At slightly acidic solution (pH=5.5) at 35°C for 1 month only minor hydrolysis of 3'-SL to sialic acid and lactose was observed. However, under acidic conditions (at pH 3.0, 35°C for 1 month or at 0.1 N HCl, 35°C for 24 hours), 3'-SL almost completely hydrolysed to sialic acid and lactose. While, under basic conditions (at pH 9.0, 35°C for 1 month or at 0.01 N NaOH 35°C for 24 hours) significant (15 to 50%) isomerization to 3'-sialyl-lactulose was noticed.

Figure B.2.2-3 Degradation Pathways of 3'-SL in Aqueous Solutions



3'-SL = 3'-sialyllactose.

B.2.3 Stability Under the Intended Conditions of Use

The stability of 2'-FL/DFL, LNT, 6'-SL sodium salt and 3'-SL sodium salt in powdered infant formula has been investigated using a high-performance liquid chromatography (HPLC) method with fluorescent detection following long-term storage for up to 30 months at various temperatures (4, 20, 30, and 37 °C).

The infant formula powder tested was a whey-based commercially available starter formula supplemented with all 5 HiMOs in combination (*i.e.*, 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt). Briefly, the HiMOs were added in the wet mixture together with other ingredients (salts, carbohydrates, proteins). Following dissolution of the ingredients, the mixtures were heat-treated at a temperature of 105 °C for 5 seconds. Subsequent steps consisted of evaporation, homogenization, and spray drying to produce a powdered product. The infant formula also contained long-chain polyunsaturated fatty acids, as well as vitamins and minerals at concentrations intended for full nutritional support of infants from birth to 6 months of age. The results of the study are presented in Table B.2.3-1. There is good alignment of the 2'-FL, DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt concentrations compared to targeted addition levels over the sampling period, which demonstrates good stability of the ingredients in powdered infant formula after 30 months storage under the tested conditions. The analytical reports for this study (which are confidential and proprietary) can be provided upon request.

Table B.2.3-1 Results of Stability of 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3'-SL Sodium Salt in Commercially Representative Infant Formula Following Storage for up to 30 Months at Various Temperatures

Temperature	Target*	T0	Sample Time (Months)					
	g/100 g IF		3	6	9	12	24	30
2'-FL								
4 °C	1.77	1.84	1.88	1.84	1.98	2.00	1.94	1.90
20 °C			1.94	1.90	1.92	1.95	1.98	1.89
30 °C			1.82	1.88	1.99	1.98	1.85	1.88
37 °C			1.86	1.86	1.88	1.94	1.83	1.90
DFL								
4 °C	0.25	0.24	0.24	0.24	0.25	0.25	0.26	0.26
20 °C			0.25	0.25	0.25	0.25	0.27	0.26
30 °C			0.23	0.24	0.26	0.25	0.25	0.26
37 °C			0.24	0.24	0.24	0.25	0.25	0.26
LNT								
4 °C	0.74	0.85	0.85	0.84	0.94	0.88	0.89	0.88
20 °C			0.85	0.87	0.92	0.88	0.89	0.88
30 °C			0.84	0.85	0.90	0.89	0.86	0.87
37 °C			0.84	0.85	0.90	0.88	0.88	0.88
6'-SL Sodium Salt								
4 °C	0.38	0.42	0.38	0.43	0.49	0.38	0.44	0.43
20 °C			0.39	0.37	0.49	0.37	0.40	0.41
30 °C			0.40	0.38	0.40	0.38	0.40	0.42
37 °C			0.40	0.38	0.45	0.36	0.41	0.41
3'-SL Sodium Salt								
4 °C	0.215	0.26	0.24	0.26	0.29	0.23	0.22	0.22
20 °C			0.23	0.23	0.29	0.23	0.21	0.23
30 °C			0.24	0.23	0.25	0.24	0.22	0.22
37 °C			0.24	0.23	0.27	0.23	0.21	0.22

2'-FL = 2'-fucosyllactose; 2'-FL/DFL = 2'-fucosyllactose and difucosyllactose mixture; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; DFL = difucosyllactose; IF = infant formula; LNT = lacto-*N*-tetraose; T0 = baseline.
* Targeted concentration of human milk oligosaccharide per 100 g of IF.

B.3 Impurity Profile

Whilst Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are predominantly purified ingredients, they also contain small quantities of D-lactose (starting substrate) and other related and fully characterised carbohydrates produced during the fermentation process. In addition to these carbohydrate-type by-products, quality control measures include the precautionary confirmation of the absence of a range of potential residual compounds and trace elements. These include amino acids and biogenic amines, as well as residual anions, trace elements, and heavy metals. Additionally, analyses have been conducted to confirm the absence of the production strain and metabolites thereof (residual proteins, DNA, and endotoxins). Details of these analyses are presented in Sections B.3.1 to B.3.4 below.

B.3.1 Absence of Amino Acids and Biogenic Amines

2'-FL/DFL, LNT, 6'-SL, and 3'-SL are secreted into the fermentation broth, and the production organism is efficiently removed during upstream processing (USP). Nevertheless, as a precautionary measure, production batches have been analysed for secondary metabolites and cellular components that may potentially originate

from the fermentation medium. No detectable levels of biogenic amines (*e.g.*, histamine, tyramine, spermidine, cadaverine and putrescine), and amino acids and their metabolites [*e.g.*, glutamic acid, *gamma*-aminobutyric acid (GABA)] have been identified in Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt ingredients. Therefore, these compounds do not contribute to the overall compositional data of the final products. The Certificates of Analysis demonstrating the absence of these parameters can be provided upon request.

B.3.2 Absence of Production Organism and its DNA

The production microorganism is efficiently removed by the ultrafiltration during USP, which is applied directly after fermentation. Additionally, during downstream processing (DSP), various sequential filtration and purification processes are applied to ensure the final purity of the 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt ingredients. Product specifications for Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt include acceptable limits for residual protein (≤ 0.01 w/w %) and residual endotoxins (≤ 10 EU/mg) (see Section B.5.1 of this Part of the application). Results from batch analyses demonstrate that the levels of residual protein and endotoxins are well below the specification for all batches of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt tested (see Section B.5.2 of this Part of the application).

The absence of the production microorganism in the bulk ingredient is demonstrated by the testing of final batches for bacteria from the *Enterobacteriaceae* family according to an internationally recognised method (ISO 21528-1).

The absence of the production organism in the finished ingredient is also supported by analyses for residual DNA in final production batches of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt. The absence of residual DNA from the production organism is confirmed by three different validated quantitative polymerase chain reaction (qPCR) methods. These qPCR methods target short sub-sequences of the inserted genes from donor organisms encoding enzymes for 2'-FL and DFL, LNT, 6'-SL, or 3'-SL biosynthesis as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. Analysis of five batches of the final 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt demonstrate no detectable levels of residual DNA (limit of quantification of 4 µg/kg) in the final ingredients (data available upon request).

B.3.3 Residual Anions and Trace Elements

Due to the nature of the fermentation process, 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt may potentially contain trace elements and minerals (as carry-over from the fermentation medium). However, the use of nanofiltration and ion-exchange purification is sufficient to reduce any appreciable carry-over of minerals from fermentation into the final ingredient. The results of trace element analyses of five batches of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium show that these do not contribute to any significant degree to the composition of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium and are therefore not proposed to be added to their specifications. Results of analysis demonstrating the absence for residual anions and trace elements can be provided upon request.

It should be highlighted that the finished food products containing 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt will comply with the mineral and compositional requirements set forth in the Australia New Zealand Food Standards Code. For instance, infant formula containing 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt to be marketed in Australia or New Zealand will conform to the compositional requirements as established in Standard 2.9.1 for infant formula products.

B.3.4 Heavy Metals

Analyses for heavy metals were conducted on 5 manufacturing batches of Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt. Specifically, results demonstrate that lead, arsenic, cadmium, and mercury are below specification limits prescribed in Schedule 3 under Section S3—4 of the Code. Certificates of analysis for heavy metal batch analyses are provided in Appendix V **(Confidential Commercial Information)**.

B.4 Manufacturing Process

B.4.1 Overview

Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are manufactured in compliance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The production process is similar among the 4 HiMO products and is largely comparable to the production process of previously authorised HiMOs (*i.e.*, 2'-FL and LNnT). A schematic overview of the manufacturing process for 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt is presented in Table B.4.1-1 below. The manufacturing process can be broadly divided into 2 stages.

In Stage 1 (USP), D-lactose (substrate) is converted to 2'-FL/DFL, LNT, 6'-SL, or 3'-SL by the adapted cellular metabolism of the 2'-FL/DFL, LNT, 6'-SL sodium salt, or 3'-SL sodium salt production microorganisms, respectively, which use D-glucose (or optional glycerol or sucrose) as an exclusive energy and carbon source. The schematic pathways for 2'-FL/DFL, LNT, 6'-SL, and 3'-SL biosynthesis are depicted in Part 3.5.1, Section A.2.2 of this application. Fermentation is maintained for several days until in-process quality controls indicate a favourable titre of 2'-FL/DFL, LNT, 6'-SL, or 3'-SL which are released into the fermentation broth, as well as high consumption of D-lactose. At the end of the fermentation process, the production microorganism cells are removed intact, without disruption, from the fermentation broth by ultrafiltration and deactivated by heat treatment in compliance with the national permit according to Directive 2009/41/EC on the contained use of genetically modified microorganisms. The permeate containing 2'-FL/DFL, LNT, 6'-SL, or 3'-SL is assessed by a range of in-process quality controls and then further purified in Stage 2 of the production process.

In Stage 2 (DSP), a series of purification, isolation and concentration steps are used to generate the final high-purity 2'-FL/DFL, LNT, 6'-SL sodium salt, or 3'-SL sodium salt products. During this stage, water, minerals, proteins, coloured bodies, charged molecules, and other small molecules and potential impurities are removed by a series of filtration steps using various filtration techniques. The concentrated 2'-FL/DFL, LNT, 6'-SL sodium salt, or 3'-SL sodium salt is subsequently dried and sampled for quality control measures and packaged.

Each of these stages are described further in Appendix VI (**Confidential Commercial Information**). To note, Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt will not be manufactured in Australia or New Zealand. Thus, the production organism used for their manufacture will not enter the territory. 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are manufactured in Denmark under contained use of the genetically modified microorganism (following strictly the Danish implementation of Directive 2009/41/EC of the European Parliament and of the Council of 06 May 2009 on the contained use of genetically modified microorganisms).

Table B.4.1-1 Overview of the Manufacturing Process for 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt

STAGE 1		**Upstream Processing (USP)**
STEPS	**1**	Media Preparation
	2	Propagation
	3	Seed Fermentation
	4	Fermentation Phases:
	4A	Growth (Batch) Phase[a]
	4B	Feeding (Fed-Batch) Phase
	4C	Harvest/Storage of Culture Broth
	5	Removal of Microorganism*
STAGE 2		**Downstream Processing (DSP)**
STEPS	**6**	Purification/Concentration 1*
	7	Ion Removal
	8	Decolourisation*
	9	Purification/Concentration 2*
	10	Drying
	11	Sampling and Packaging

Table B.4.1-1 Overview of the Manufacturing Process for 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt

STAGE 1		Upstream Processing (USP)
	12	Quality Control and Batch Release

2'-FL/DFL = 2'-fucosyllactose/difucosyllactose mixture; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; LNT = lacto-*N*-tetraose.

[a] The batch phase of fermentation is optional.

* After the marked steps additional sterile filtration (microfiltration) is performed to maintain low microbial load during all times of downstream processing and to ensure high microbial quality of the final ingredient. These steps provide further reassurance for the absence of the production microorganism in final ingredient.

B.4.2 Identity of Raw Materials and Processing Aids

The raw materials used to synthesise 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are food-grade D-lactose as the substrate and D-glucose (or alternatively glycerol or sucrose) as the energy and carbon source. These raw materials are sterilised before use. Fermentation is performed in a chemically defined, salt-based, minimal growth medium with D-glucose (or alternatively glycerol or 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 considering a range of strict food quality requirements. Raw materials and processing aids used in the production of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt by fermentation are provided in Appendix VI **(Confidential Commercial Information)**, along with their functions in the production process.

B.4.3 Quality Control

As previously indicated, the manufacture of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt by microbial fermentation is conducted in accordance with cGMP and HACCP principles. Due to the fact that the final products are simple mixtures of well-characterised and pure compounds, the whole production process can be followed in detail by a range of analytical techniques. These techniques are applied either as in-process controls (detailed in Appendix VI) or at batch release (by Certificate of Analysis) to allow full control of the production process.

Both manufacturing stages (USP and DSP) are controlled by a HACCP plan which includes specifications for equipment, raw materials, product, and packaging materials. Master operating instructions are followed, batch records are kept, a number of in-process controls are applied, and the isolated product is controlled by Certificates of Analysis and batch release routines.

The HACCP plan for both manufacturing stages comprises a number of in-process controls to minimise the amount of potential inherent impurities to the level technically possible. The Glycom production process (including all used processing aids, raw materials, unit operations and filter aids) as well as the food safety management system comply with the Food Safety Systems Certification 22000 (FSSC 22000) and the 2015 International Organisation for Standardisation 9001 (ISO 9001:2015).

B.5 Specification for Identity and Purity

B.5.1 Product Specifications

Food grade specifications for Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are presented in the sections that follow. As mentioned, Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt have been accepted for use under similar specifications in other jurisdictions such as the EU (EU, 2019, 2020, 2021a,b) and the U.S. (GRNs 815, 833, 880 and 881 – U.S. FDA, 2019a,b, 2020a,b). All methods of analysis are either internationally recognised or developed and validated internally by Glycom and confirmed by independent accredited external laboratories. Details of the internal methods of analysis are provided in Appendix VII **(Confidential Commercial Information)**.

In addition to specifications confirming the purity of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt, upper limits have also been established for the raw material used in the manufacturing process (D-lactose), the

carbohydrates formed during the fermentation, trace elements, heavy metals, and microbiological parameters to ensure the purity of the final product.

Notably, as residual lactose (disaccharide) and some of the carbohydrates formed during fermentation are naturally occurring components of human milk, 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt each have a specification for human-identical milk saccharides (HiMS) to capture the total fraction of carbohydrates naturally occurring in human milk. Minor amounts of other related and fully characterised carbohydrates are captured under a specification called "sum of other carbohydrates".

2'-FL/DFL

Specifications for Glycom's 2'-FL/DFL are presented in Table B.5.1-1 below. The 2'-FL/DFL product is specified as containing at least 85.0 % of 2'-FL and DFL on a dry weight basis. Individually, 2'-FL is specified to be at least 75.0 % (water-free), and DFL is specified to be at a minimum of 5.0 % (water-free).

Table B.5.1-1 Product Specifications for 2'-FL/DFL

Parameter	Specification	Method
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Colour	White, white to off-white, off-white	ISO 6658
Identification by RT	RT (main components) corresponds to RT (standards) ± 3%	Glycom method HPLC-202-2C4-002 and HPAEC-HMO-011
Assay (water-free) HiMS[a]	≥ 92.0 w/w %	Glycom method HPLC-202-2C4-002 and HPAEC-HMO-011
Assay (water-free) Sum of 2'-FL and DFL	≥ 85.0 w/w %	Glycom method HPLC-202-2C4-002 and HPAEC-HMO-011
Assay (water-free) 2'-Fucosyllactose	≥ 75.0 w/w %	Glycom method HPLC-202-2C4-002 and HPAEC-HMO-011
Assay (water-free) Difucosyllactose	≥ 5.0 w/w %	Glycom method HPAEC-HMO-011
D-Lactose	≤ 10.0 w/w %	Glycom method HPAEC-HMO-011
L-Fucose	≤ 1.0 w/w %	Glycom method HPLC-2-003
2'-Fucosyl-D-lactulose	≤ 2.0 w/w %	Glycom method HPLC-2-003
Sum of other carbohydrates	≤ 6.0 w/w %	Glycom methods: HPAEC-HMO-011 and HPLC-2-003
pH in 5 % solution (20 °C)	4.0 - 6.0	Eur. Ph. 2.2.3
Water	≤ 6.0 w/w %	Glycom method KF-001
Ash, sulphated	≤ 0.8 w/w %	Eur. Ph. 2.4.14
Residual protein by Bradford assay	≤ 0.01 w/w %	Glycom method UV-001
Residual endotoxins	≤ 10 E.U./mg	Eur. Ph. 2.6.14 (LAL kinetic chromogenic assay)
Lead	≤ 0.1 mg/kg	EN 13805, EPA-6020A
Microbiological Parameters		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae	Absent in 10 g	ISO 21528-1
Salmonella spp.	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Yeasts	≤ 100 CFU/g	ISO 21527-2
Moulds	≤ 100 CFU/g	ISO 21527-2

2'-FL = 2'-fucosyllactose; 2'-FL/DFL = 2'-fucosyllactose and difucosyllactose mixture; 3-FL = 3-fucosyllactose; AFNOR = Association Francaise de Normalisation; CFU = colony forming units; DFL = difucosyllactose; EN = European Standards; Eur. Ph. = European Pharmacopeia; E.U. = endotoxin units; HiMS = human-identical milk saccharides; HPAEC = high-performance anion-exchange chromatography; ISO = International Organization for Standardization; KF = Karl-Fischer; NMKL = Nordisk Metodikkomite for Levnedsmidler; RT = retention time.
[a] HiMS include 2'-FL, DFL, 3-FL, D-lactose and L-fucose.

LNT

Specifications for Glycom's LNT are presented in Table B.5.1-2 below. The LNT product is specified as containing at least 70 % of LNT on a dry weight basis.

Table B.5.1-2 Product Specifications for LNT

Parameter	Specification	Method
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Colour	White, white to off-white, off-white	ISO 6658
Identification by Retention Time	RT of main component corresponds to RT of standard ± 3%	Glycom method HPLC-7-001
Assay (water-free) HiMS[a]	≥ 90.0 w/w %	Glycom methods HPLC-7-001, HPAEC-HMO-006
Assay (water free) LNT	≥ 70.0 w/w %	Glycom methods HPLC-7-001, HPAEC-HMO-006
D-lactose	≤ 12.0 w/w %	Glycom method HPAEC-HMO-006
Lacto-*N*-triose II	≤ 10.0 w/w %	Glycom method HPAEC-HMO-006
para-lacto-*N*-hexaose-2	≤ 3.5 w/w %	Glycom method HPAEC-HMO-006
LNT fructose isomer	≤ 1.0 w/w %	Glycom method HPLC-7-002
Sum of other carbohydrates	≤ 5.0 w/w %	Glycom method HPLC-7-002, HPAEC-HMO-006
pH in 5% solution (20 °C)	4.0 to 6.0	Ph. Eur. 2.2.3
Water	≤ 6.0 w/w %	Glycom method KF-001
Ash, sulphated	≤ 0.5 w/w %	Ph. Eur. 2.4.14
Residual protein by Bradford assay	≤ 0.01 w/w %	Glycom method UV-001
Residual endotoxins	≤ 10 EU/mg	Ph. Eur. 2.6.14
Lead	≤ 0.1 mg/kg	EN 13805:2002, EPA-6020A:2007
Microbiological Parameters		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae	Absent in 10 g	ISO 21528-1
Salmonella spp.	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Yeasts	≤ 100 CFU/g	ISO 21527-2
Moulds	≤ 100 CFU/g	ISO 21527-2

AFNOR = Association Française de Normalisation; CFU = colony forming units; EN = European Standards; EPA = Environmental Protection Agency; EU = endotoxin units; HiMS = human-identical milk saccharides; HPAEC = high-performance anion exchange chromatography; HPLC = high-performance liquid chromatography; ISO = International Organization for Standardization; KF = Karl Fischer; LNT = lacto-*N*-tetraose; NMKL = Nordisk Metodikkomite for Levnedsmidler; Ph. Eur. = European Pharmacopoeia; RT = retention time; UV = ultraviolet.
[a] Sum of LNT, D-lactose, and lacto-*N*-triose II.

6'-SL sodium salt

Specifications for Glycom's 6'-SL sodium salt are presented in Table B.5.1-3 below. The 6'-SL sodium salt product is specified as containing at least 90.0 % of 6'-SL sodium salt on a dry weight basis.

Table B.5.1-3 Product Specifications for 6'-SL Sodium Salt

Parameter	Specification	Method
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Colour	White, white to off-white, off-white	ISO 6658
Identification	RT of main component corresponds to RT of standard ± 3%	Glycom method HPAEC-HMO-009
Assay (water-free) HiMS[a]	≥ 94.0 w/w %	Glycom method HPAEC-HMO-009, HPAEC-HMO-010
Assay (water free) 6'-SL sodium salt	≥ 90.0 w/w %	Glycom method HPAEC-HMO-009
D-lactose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-010
Sialic acid	≤ 2.0 w/w %	Glycom method HPAEC-HMO-009
6'-Sialyl-lactulose	≤ 3.0 w/w %	Glycom method HPAEC-HMO-009
Sum of other carbohydrates	≤ 3.0 w/w %	Glycom method HPAEC-HMO-009, HPAEC-HMO-010
Sodium	2.5 – 4.5 w/w %	EN 13805:2002; EPA-6010C:2007 or GOST 31869-2012
Chloride	≤ 1.0 w/w %	GOST 31867-2012
pH in 5% solution (20 °C)	4.5 – 6.0	Ph. Eur. 2.2.3
Water	≤ 6.0 w/w %	Glycom method KF-002
Residual protein by Bradford assay	≤ 0.01 w/w%	Glycom method UV-003
Residual endotoxins	≤ 10 EU/mg	Ph. Eur. 2.6.14 (LAL kinetic chromogenic assay)
Lead	≤ 0.1 mg/kg	EN 13805:2002; EPA-6020A:2007
Microbiological Parameters		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae	Absent in 10 g	ISO 21528-1
Salmonella spp.	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Yeasts	≤ 100 CFU/g	ISO 21527-2
Moulds	≤ 100 CFU/g	ISO 21527-2

6'-SL = 6'-sialyllactose; AFNOR = Association Française de Normalisation; CFU = colony forming units; EN = European Standards; EPA = Environmental Protection Agency; EU = endotoxin units; HiMS = human-identical milk saccharides; HPAEC = high-performance anion exchange chromatography; ISO = International Organization for Standardization; KF = Karl Fischer; NMKL = Nordisk Metodikkomite for Levnedsmidler; Ph. Eur. = European Pharmacopoeia; RT = retention time; UV = ultraviolet.

[a] Sum of 6'-SL sodium salt, D-lactose, and sialic acid.

3'-SL sodium salt

Specifications for Glycom's 3'-SL sodium salt are presented in Table B.5.1-4 below. The 3'-SL sodium salt product is specified as containing at least 88.0 % of 3'-SL sodium salt on a dry weight basis.

Table B.5.1-4 Product Specifications for 3'-SL Sodium Salt

Parameter	Specification	Method
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Colour	White, white to off-white, off-white	ISO 6658
Identification	RT of main component corresponds to RT of standard ± 3%	Glycom method HPAEC-HMO-007
Assay (water-free) HiMS[a]	≥ 90.0 w/w %	Glycom method HPAEC-HMO-007, HPAEC-HMO-008
Assay (water free) 3'-SL sodium salt	≥ 88.0 w/w %	Glycom method HPAEC-HMO-007
D-lactose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-008
Sialic acid	≤ 1.5 w/w %	Glycom method HPAEC-HMO-007
3'-Sialyl-lactulose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-007
Sum of other carbohydrates	≤ 3.0 w/w %	Glycom method HPAEC-HMO-007, HPAEC-HMO-008
Sodium	2.5 – 4.5 w/w %	GOST 31869-2012
Chloride	≤ 1.0 w/w %	GOST 31867-2012
pH in 5% solution (20 °C)	4.5 – 6.0	Ph. Eur. 2.2.3
Water	≤ 8.0 w/w %	Glycom method KF-002
Residual protein by Bradford assay	≤ 0.01 w/w%	Glycom method UV-003
Residual endotoxins	≤ 10 EU/mg	Ph. Eur. 2.6.14 (LAL kinetic chromogenic assay)
Lead	≤ 0.1 mg/kg	EN 13805:2002; EPA-6020A:2007
Microbiological Parameters		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae	Absent in 10 g	ISO 21528-1
Salmonella	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Yeasts	≤ 100 CFU/g	ISO 21527-2
Moulds	≤ 100 CFU/g	ISO 21527-2

3'-SL = 3'-sialyllactose; AFNOR = Association Française de Normalisation; CFU = colony forming units; EN = European Standards; EPA = Environmental Protection Agency; EU = endotoxin units; HiMS = human-identical milk saccharides; HPAEC = high-performance anion exchange chromatography; ISO = International Organization for Standardization; KF = Karl Fischer; NMKL = Nordisk Metodikkomite for Levnedsmidler; Ph. Eur. = European Pharmacopoeia; RT = retention time; UV = ultraviolet.

[a] Sum of 3'-SL sodium salt, D-lactose, and sialic acid.

B.5.2 Batch Analyses

Analyses have been conducted on 5 independent representative batches of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt. Certificates of Analysis are provided in Appendix V **(Confidential Commercial Information)**. The results demonstrate that 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt comply with the established product specifications defined in Section B.5.1.

B.6 Analytical Method for Detection

The presence of 2'-FL, DFL, LNT, 6'-SL, 3'-SL in milk products can be detected and quantified using an analytical method involving liquid chromatography, based on the methodology described by Austin and Bénet (2018).

B.7 Proposed Food Label

Infant formula products containing 2'-FL/DFL, LNT, 6'-SL sodium salt, and/or 3'-SL sodium salt will be labelled with a statement of ingredients according to general requirement in Standard 1.2.4 of the Code. The ingredients are proposed to be declared in the statement of ingredients according to labelling provisions in Section 1.2.4—4, requiring ingredients to be identified using a name by which they are commonly known or a name that describes its true nature.

In addition to the general labelling requirements established under Part 1.2 of the Code, infant formula products containing 2'-FL/DFL, LNT, 6'-SL sodium salt, and/or 3'-SL sodium salt will be labelled in accordance with the specific provisions established in Standard 2.9.1 of the Code. As 2'-FL/DFL, LNT, 6'-SL sodium salt, and/or 3'-SL sodium salt are intended to be added as nutritive substances to infant formula products, the average amount for each is proposed to be declared on the nutrition information statement of infant formula products containing the ingredient in accordance to Section 2.9.1—21(1)(a)(iii) of the Code. Furthermore, as per prohibitions in Section 2.9.1—24(ca) or (cb) of the Code, the words 'human milk oligosaccharide' or 'human milk identical oligosaccharide', and the abbreviations 'HMO' or 'HiMO', and any other word(s) or abbreviation having the same or similar effect, will not be used to identify 2'-FL/DFL, LNT, 6'-SL sodium salt, and/or 3'-SL sodium salt on the statement of ingredients, or be used anywhere else on the label or package.

C. Information Related to the Safety of the Nutritive Substance

Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt ingredients are considered foods produced using gene technology used for a nutritive purpose in special purpose foods (infant formula products). The general basis of the approach of this safety assessment is the demonstration that:

(1) The 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced using gene technology are well characterised and confirmed to be chemically and structurally identical to their natural counterparts found in human milk.

(2) These ingredients will be added at levels such that the levels of the specified HiMOs (*i.e.,* 2'-FL, DFL, LNT, 6'-SL, and 3'-SL) will not exceed those encountered in human milk (on an individual and total basis).

(3) 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are highly purified ingredients.

(4) The production strain and genetic material is removed from the finished ingredient.

(5) Corroborative safety studies, including those conducted in animals and humans, demonstrate that these ingredients do not pose any safety concerns.

(6) Glycom's 2'-FL and LNnT produced using the same platform strain have been approved for use in infant formula products in Australia/New Zealand.

(7) Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt have been approved for use and commercialised in infant formula products in other jurisdictions.

C.1 Toxicokinetics and Metabolism, Degradation Products, and Major Metabolites

The manufactured 2'-FL, DFL, LNT, 6'-SL, and 3'-SL molecules are chemically and structurally identical to their naturally occurring counterparts in human milk (see Appendix III). Absorption, distribution, metabolism, and excretion/elimination (ADME) of HMOs have been investigated in a number of studies (Brand-Miller *et al.*, 1995, 1998; Rudloff *et al*., 1996, 2006, 2012; Obermeier *et al*., 1999; Engfer *et al.*, 2000; Gnoth *et al*., 2000; Chaturvedi *et al*., 2001; Coppa *et al*., 2001; Rudloff and Kunz, 2012; De Leoz *et al*., 2013; Dotz *et al*., 2014, 2015; Goehring *et al*., 2014; Kunz and Rudloff, 2017). These studies suggest that HMOs do not undergo any significant digestion in the upper gastrointestinal tract but are instead fermented in the colon by intestinal microbiota or are excreted unchanged in the faeces. However, a small proportion of ingested HMOs may be absorbed intact, with approximately 1 to 2 % of the total amount of HMO ingested being excreted unchanged in the urine. The absorption of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL is expected to be highly limited and any level that is absorbed would be no different to that occurring in breastfed infants.

Consistent with these publications, in EFSA's Scientific Opinion on the essential composition of infant and follow-on formula, the EFSA NDA Panel considers HMOs as "non-digestible oligosaccharides" (EFSA, 2014). Furthermore, in their Scientific Opinions on the safety of 2'-FL/DFL mixture, LNT, 6'-SL sodium salt, and 3'-SL sodium salt, it was reiterated that the EFSA NDA Panel "*considers that limited digestion of the NF [novel food] occurs in the upper gastrointestinal tract and that only small amounts are expected to be absorbed*" (EFSA, 2019a,b; EFSA, 2020a,b).

C.2 Animal or Human Studies

C.2.1 Toxicological Data

The risk assessment approach for 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt follows the same procedures used to support the safety of these HiMO ingredients and others that have received authorisations in the EU, the U.S., and elsewhere (*e.g.*, Brazil, Israel, Malaysia, Singapore, Thailand, and the UK). Pivotal data and information supporting the safety of Glycom's HiMO products are based on qualitative and quantitative data establishing that these HiMOs are chemically and structurally identical to those present within human milk, and that they are intended for use in infant formula products at levels equivalent to mean levels that have been reported for human milk samples across all lactational stages. The safety of non-HiMO constituents originating from the fermentation organism is supported in part by the general history of safe use of *E. coli* K-12 for the production of food ingredients (*i.e*., *E. coli* K-12 and its derivatives are non-pathogenic and non-toxigenic strains) as well as specification limits for potential undesirable substances (such as residual proteins and endotoxins), and is further supported by results of animal toxicity studies conducted on HiMO preparations produced using derivatives of Glycom's platform strain.

Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt have each been tested in a comprehensive series of toxicological studies, including a bacterial reverse mutation assay, an *in vitro* mammalian cell micronucleus test in human lymphocytes, and an adapted sub-chronic (90-day) oral toxicity study in neonatal rats. In all studies, test articles were representative of the material intended to be commercially marketed. The studies were performed in accordance with the Organisation for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) and appropriate OECD test guidelines. Notably, the adapted study design of the sub-chronic (90-day) oral toxicity study incorporating the use of neonatal animals considers the requirements of EFSA Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (EFSA, 2017), Guidance for industry: nonclinical safety evaluation of paediatric drug products (U.S. FDA, 2006), Guideline on the need for non-clinical testing in juvenile animals of pharmaceuticals for paediatric indications (EMEA, 2008), and the Guideline on the Nonclinical Safety Study in Juvenile Animals for Paediatric Drugs (MHLW, 2018). Detailed descriptions of these studies are presented in Sections that follow. The toxicological studies conducted with Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and

3'-SL sodium salt have been published (Phipps *et al*., 2018a,b; Phipps *et al*., 2019a,b), while a copy of the full unpublished study reports is provided in Appendix VIII **(Confidential Commercial Information)**.

In addition to the product-specific studies conducted with Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt, a tabular summary of toxicological studies that have been conducted with other relevant HiMO preparations (including HiMO mixtures) considered relevant to this application is provided in Annex I.

C.2.1.1 Studies Conducted with Glycom's 2'-FL/DFL

Repeated-Dose Studies

14-Day Toxicity Study in the Neonatal Rat

A 14-day repeat dose toxicity study was conducted in rats to evaluate the potential short-term toxicity of 2'-FL/DFL and select dose levels for the subsequent 90-day study (Flaxmer, 2018a [unpublished]). The test article (Batch CPN6317 1000517 FD) was composed of 82.5 w/w % 2'-FL and 9.7 w/w % DFL (equivalent to 92.2 w/w % 2'-FL/DFL).

Groups of 8 male and 8 female neonatal rats (post-natal day 7) were dosed with 0 (water for irrigation), 4,000, or 5,000 mg/kg body weight/day 2'-FL/DFL, by gavage at a dose volume 10 mL/kg body weight, once daily for 14 days, until the day before necropsy. Doses of 2'-FL/DFL were corrected to account for "other carbohydrates" within the test article batches, thus the nominal values (*i.e.,* 4,000 and 5,000 mg/kg body weight/day) reflect the actual 2'-FL and DFL content. The total dosages of the test article administered were 4,368 and 5,460 mg/kg body weight/day.

All animals were observed daily for changes in clinical condition. Body weights were recorded daily until the end of the dosing period, when animals were subjected to a gross macroscopic necropsy.

There were no test item-related deaths. One male receiving 5,000 mg/kg body weight/day was found dead on Day 15 (just prior to scheduled necropsy). The precise cause of death is unclear; no clinical signs had been observed for this animal. The animal gained a similar amount of weight to other males throughout the study and macroscopic examination revealed no abnormalities, including no evidence of dosing trauma. However, in the absence of any other deaths during the study, this isolated death was considered incidental and unrelated to the test item.

Transient clinical signs (red and/or yellow staining around the anus was reported for some 2'-FL/DFL-treated animals) were absent by the end of the treatment period and were considered non-adverse. There were no biologically relevant differences in body weight between test item-treated groups and controls. No test item-related macroscopic abnormalities were reported.

In the absence of any test item-related adverse findings, 5,000 mg 2'-FL/DFL/kg body weight/day (the maximum tolerated dose, based on data for similar compounds) was considered the no-observed-adverse-effect level (NOAEL) and a suitable high-dose for the 90-day study.

90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential sub-chronic toxicity of 2'-FL/DFL (Batch CPN6317 1000517 FD) when administered by gavage to neonatal rats from Day 7 of age (Flaxmer, 2018b [unpublished]); Phipps *et al.*, 2018a]. The study was conducted in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b) with an adaptation for the use of neonatal animals.

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 1,000, 3,000, or 5,000 mg/kg body weight/day 2'-FL/DFL (doses corrected to account for other carbohydrates), by gavage at a dose volume of 10 mL/kg body weight, once daily for at least 90 days, until the day before necropsy. An additional reference control group (comprising the same number of animals) received fructo-oligosaccharide (FOS), a non-digestible oligosaccharide permitted in infant nutrition, at 5,000 mg/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose 2'-FL/DFL group and identify any effects related to the general fibre-like characteristics of the reference material. A further 5 males and 5 females in each group were also dosed once daily for at least 90 days and then kept undosed for 4 weeks, to

assess the reversibility of any observed effects. These dosages represent the amount of 2'-FL/DFL and the FOS reference control that were administered. To account for the “other carbohydrates” that are present in the test articles, the dosages were adjusted so that the total amount of test articles administered were 1,092, 3,276, and 5,460 mg/kg body weight/day for the groups receiving 2'-FL/DFL, and 5,320 mg/kg body weight/day for the oligofructose reference control.

Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of treatment until weaning and twice weekly thereafter. Food intake was recorded twice weekly from weaning until necropsy. The eyes of vehicle control, reference control, and high-dose animals were examined in Week 13. Blood samples were taken for haematology, blood chemistry, and coagulation during Week 13 and at the end of the treatment-free period; urine samples were collected for urinalysis in Week 13 and at the end of the treatment-free period.

In Week 11 or 12, all animals were subjected to a functional observational battery consisting of observations in-hand and in a standard area, in addition to an assessment of grip strength and learning and memory (using the Morris water maze). Pre-weaning reflex development (eye opening, air righting, startle response, and pupil closure response), ulna length and sexual maturation (balano-preputial separation and vaginal opening) were also recorded for all animals during the treatment period.

All animals at the end of the treatment and recovery periods were subjected to a gross macroscopic necropsy, where selected organs (adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus, thyroid with parathyroids, and uterus with cervix) were weighed and fixed. The following tissues were examined microscopically: adrenals, brain, femur, heart, kidneys, liver, lungs, nasal turbinates, spinal cord, sternum, stomach, thyroid, and uterus) for animals in the vehicle control and high-dose 2'-FL/DFL groups.

There were no deaths and no test item-related clinical signs or ocular changes. Animals given 2'-FL/DFL gained similar amounts of weight and ate similar amounts of food compared with controls. There was no effect of 2'-FL/DFL administration on pre-weaning development [as evaluated by the age of attainment of the surface and air righting reflexes and the pupil reflex and startle response tests conducted on Day 14 of treatment (Day 20 of age)]. Ulna length and growth were similar between 2'-FL/DFL-treated groups and controls. No test item-related differences in behaviour of the animals during the in-hand and arena observations in Week 11 of treatment (Day 81 to 83 of age) were reported. Mean activity count for females given 5,000 mg/kg body weight/day was slightly lower than that of vehicle controls, but as this was not seen for males and there was no dose response-relationship, it was considered unrelated to the administration of 2'-FL/DFL; the mean value (19.8) was also similar to the value for reference controls (20.6). Morris maze performance was also unaffected by administration of 2'-FL/DFL, with clear evidence of learning and memory over the 4 days of testing, as demonstrated by generally progressive decreases in group mean trial times, sector entries, and failed trials.

There were no test item-related differences for the mean body weight or day of age at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation and vaginal opening for males and females, respectively). Mean age and body weight for balano-preputial skinfold separation were slightly higher for 2'-FL/DFL-treated males from the high-dose group compared with vehicle controls. However, the differences were minor and considered to be due to aberrantly low vehicle control values, rather than any effect of the test item; four individual vehicle control values for age and four for body weight were below the 5 % confidence historical control data (HCD) limits, compared to only one individual high-dose 2'-FL/DFL value (for either parameter) being outside the respective HCD ranges. Furthermore, the 5,000 mg/kg body weight/day 2'-FL/DFL values were comparable to reference control values.

No test item-related or biologically relevant differences in haematology parameters between 2'-FL/DFL-treated groups and controls were reported. The statistically significant differences reported at the end of the treatment period were not associated with a dose response [increased haematocrit, haemoglobin, and red blood cells for males (all 2'-FL/DFL groups) and females (1,000 or 3,000 mg/kg body weight/day); increased mean cell volume for both sexes given 1,000 or 3,000 mg/kg body weight/day; decreased mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration for males (all groups) and females (1,000 or 3,000 mg/kg body weight/day); increased mean corpuscular volume for males and females at 3,000 mg/kg body weight/day; decreased red blood cell distribution width for males given 1,000 or 3,000 mg/kg body weight/day; increased basophils and large unstained cells for males (all 2'-FL/DFL groups); increased basophils for females (all 2'-FL/DFL groups); decreased white blood cell count, lymphocytes, and eosinophils for females receiving 1,000 or 3,000 mg/kg body weight/day; decreased monocytes for females (all 2'-FL/DFL groups)].

Values for all of these parameters were also within the respective HCD ranges. Statistically significantly increased prothrombin time for males in all 2'-FL/DFL groups was also not associated with a dose response and was not seen for females. Furthermore, the value for the high dose group (22.2 seconds) was similar to that for reference controls (22.9 seconds). However, some of the individual values were above the HCD upper limit (6, 5 and 3 out of 10 at 1,000, 3,000, or 5,000 mg/kg body weight/day, respectively). Although this was likely attributable to the vehicle controls having relatively low values (2 out of 10 were below the HCD lower limit), this parameter was re-evaluated at the end of the treatment-free period and values were similar between all groups.

There were also no test item-related or biologically relevant differences in blood chemistry parameters between 2'-FL/DFL-treated groups and controls. Statistically significant differences compared with vehicle controls were either not associated with a dose-response relationship [increased aspartate aminotransferase (AST) for males given 1,000 or 3,000 mg/kg body weight/day and females given 1,000 mg/kg body weight/day; reduced albumin for males given 3,000 mg/kg body weight/day and females in all 2'-FL/DFL-treated groups] or the differences were inconsistent between the sexes (decreased creatinine for males given 5,000 mg/kg body weight/day, increased urea for females receiving 1,000 or 3,000 mg/kg body weight/day and increased calcium and phosphorus for females given 5,000 mg/kg body weight/day). Values for all of these parameters were within the respective HCD ranges.

Urinalysis parameters were unaffected by 2'-FL/DFL administration. Urinary pH was statistically significantly higher for females receiving 5,000 mg/kg body weight/day 2'-FL/DFL and total creatinine was statistically significantly lower, compared with vehicle controls. However, as the differences were not seen in males and values for both parameters were within the respective HCD ranges, these findings were considered biologically irrelevant and unrelated to the administration of 2'-FL/DFL.

There were no test item-related differences in organ weights between 2'-FL/DFL-treated groups and vehicle controls at the end of the treatment or treatment-free periods. Where statistically significant differences were reported, the differences were clearly unrelated to 2'-FL/DFL administration (increased body weight adjusted kidney and seminal vesicle weights for males given 1,000 mg/kg body weight/day and increased thymus weights for all male 2'-FL/DFL-treated groups at the end of the treatment period were not dose-related; increased body weight adjusted pituitary weights for females given 5,000 mg/kg body weight/day were only seen at the end of the recovery period and were not evident immediately after cessation of dosing).

Macroscopic examinations (at the end of the treatment and treatment-free periods) and histopathological examinations (at the end of the treatment period only) revealed no test item-related findings. The only findings reported were incidental and generally consistent with changes encountered in Sprague-Dawley rats of this age kept under laboratory conditions.

In absence of any test item-related adverse effects, the NOAEL was concluded to be 5,000 mg/kg body weight/day 2'-FL/DFL, the highest dose tested and maximum tolerated dose, based on data for similar compounds.

Genotoxicity Studies

Bacterial Reverse Mutation Test

The potential mutagenicity of 2'-FL/DFL (Batch CPN6317 1000517 FD) was evaluated in a bacterial reverse mutation test (Ames test) (Phipps *et al.*, 2018a; Šoltésová, 2018a [unpublished]). This study was performed in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 471 (OECD, 1997), Commission Regulation (EC) No 440/2008[11] B13/14, U.S. Environmental Protection Agency (EPA) Health

[11] Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). OJ L 142, 31.5.2008, p. 1–739. Available at: https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32008R0440 (current consolidated version: 16/10/2019).

Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998), and FDA Redbook IV.C.1.a. (U.S. FDA, 2000).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium (*S.* Typhimurium) strains TA98, TA100, TA1535 and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were treated with 2'-FL/DFL at concentrations of up to 5,000 µg/plate (the regulatory maximum dose level) in the absence and presence of external metabolic activation (S9 mix).

Water (purified by reverse osmosis) served as the vehicle for 2'-FL/DFL and as the negative control. Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene, and 4-nitroquinoline-1-oxide) and presence (2-aminoanthracene and benzo[a]pyrene) of metabolic activation. A positive result for mutagenicity was defined as a dose-dependent and biologically relevant 2- or 3-fold increase in the number of revertant colonies, compared to that of the vehicle control group.

There was no evidence of mutagenicity in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced significant increases in revertant colony counts (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded that 2'-FL/DFL is non-mutagenic at concentrations up to 5,000 µg/plate (the regulatory maximum dose level).

In Vitro Mammalian Cell Micronucleus Test

The clastogenic and aneugenic potential of 2'-FL/DFL (Batch CPN6317 1000517 FD) was evaluated in an *in vitro* mammalian cell micronucleus test conducted using human lymphocytes (Gilby, 2018a [unpublished]; Phipps *et al.*, 2018a). This study was done in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 487 (OECD, 2016).

An initial preliminary cytotoxicity test was conducted using 2'-FL/DFL at concentrations of 0 to 2,000 µg/mL (the regulatory maximum dose level), in the presence (3-hour treatment) and absence (3 and 24-hour treatments) of S9 metabolic activation, where there was no evidence of cytotoxicity observed at any dose level. Cytotoxicity was assessed again in the main experiment, where there was no evidence of cytotoxicity at any dose level under any of the experimental conditions.

In the main experiment for micronucleus analysis, human lymphocytes were treated with concentrations of 2'-FL/DFL at 500, 1,000, or 2,000 µg/mL with S9 (3 hours) and without S9 (3 and 24-hour treatments). The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronucleated binucleated cells (MNBC), with the frequency of MNBC also being above upper historical vehicle control limit.

There was no evidence of clastogenicity or aneugenicity in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in MNBC (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded that 2'-FL/DFL is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL (the regulatory maximum dose level), in the absence and presence of metabolic activation.

C.2.1.2 Studies Conducted with Glycom's LNT

The same test article (Batch CPN4215 1000416 FD), composed of 77 % LNT on a dry weight basis, was evaluated in all studies

Repeated-Dose Studies

14-Day Toxicity Study in the Neonatal Rat

A 14-day repeat dose toxicity study was conducted in rats to evaluate the potential short-term toxicity of LNT and select dose levels for the subsequent 90-day study (Phipps *et al*., 2018b; Stannard, 2019a [unpublished]).

Groups of 8 male and 8 female neonatal rats were dosed with 0 (water for irrigation), 3,250, or 4,000 mg LNT/kg body weight/day, by gavage at a dose volume 10 mL/kg body weight, once daily for 14 days, until the

day before necropsy. The high dose of 4,000 mg LNT/kg body weight/day was the maximum feasible dose, based on viscosity of the test item formulation. Doses of LNT were corrected to account for "other carbohydrates" within the test article batch.

All animals were observed daily for changes in clinical condition. Body weights were recorded daily until the end of the dosing period, when animals were subjected to a gross macroscopic necropsy.

There were no test item-related deaths. One male receiving 4,000 mg LNT/kg body weight/day was found dead on Day 14 of dosing. This animal had shown no changes in clinical condition; however, it gained slightly less weight (2 %) than the other males in this group (8 to 11 %) between Days 13 and 14 of dosing. Macroscopic examination revealed no abnormalities and there was no evidence of dosing trauma. As this was an isolated incident, this premature death was considered incidental and unrelated to treatment with LNT. There were no test item-related clinical signs, no biologically relevant differences in body weight between test item-treated groups and controls, and no test item-related macroscopic abnormalities at necropsy.

In the absence of any test item-related adverse findings, 4,000 mg LNT/kg body weight/day (the maximum feasible dose, based on viscosity) was considered the no-observed-adverse-effect level (NOAEL) and a suitable high-dose for the 90-day study.

90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential sub-chronic toxicity of LNT when administered by gavage to neonatal rats from Day 7 of age (Phipps *et al*., 2018b; Stannard, 2018 [unpublished]). The study was conducted in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b) with an adaptation for the use of neonatal animals.

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 1,000, 2,500, or 4,000 mg LNT/kg body weight/day, by gavage at a dose volume of 10 mL/kg body weight, once daily for at least 90 days, until the day before necropsy. An additional reference control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 4,000 mg LNT/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose LNT group and identify any effects related to the general fibre-like characteristics of the reference material. A further 5 males and 5 females in each group were also dosed once daily for at least 90 days and then kept un-dosed for 4 weeks, to assess the reversibility of any observed effects.

Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of treatment until weaning and twice weekly thereafter. Food intake was recorded twice weekly from weaning until necropsy. The eyes of vehicle control, reference control and high dose LNT animals were examined in Week 13. Blood samples were taken for haematology, blood chemistry and coagulation during Week 13 and at the end of the treatment-free period. Additional blood samples were taken at the end of the treatment period for potential analysis of thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) and were stored frozen until the end of the study. Urine samples were collected for urinalysis in Week 13 and at the end of the treatment-free period.

In Week 11/12, all animals were subjected to a functional observational battery consisting of observations in-hand and in a standard area, in addition to an assessment of grip strength and learning and memory (using the Morris water maze). Pre-weaning reflex development (eye opening, air righting, startle response, and pupil closure response), ulna length, and sexual maturation (balano-preputial separation and vaginal opening) were also recorded for all animals during the treatment period.

All surviving animals (at the end of the treatment and recovery periods) were subjected to a gross macroscopic necropsy, selected organs/tissues were weighed and fixed. At the end of the treatment period, a full list of organs/tissues for early decedents and animals in the vehicle control, high-dose LNT, and reference control groups were examined microscopically.

There were no test item-related deaths. Six animals (1 male and 1 female from the vehicle control group, 1 male and 1 female given 1,000 mg LNT/kg body weight/day, 1 reference control female and 1 female given 4,000 mg LNT/kg body weight/day) died during the study, but macro- and microscopic examinations did not identify any test item-related lesions in these animals. Three of the deaths were considered to be due to dosing trauma and there was no specific cause of death reported for the other three decedents, but all were considered incidental and unrelated to treatment with LNT.

No test item-related clinical signs or ocular findings were observed. Animals administered LNT gained similar amounts of weight and consumed similar amounts of food compared with controls. Overall mean food intake for the high-dose males was statistically significantly lower than vehicle controls, but the value (23 g/animal/day) was exactly the same as reported for males provided LNT at a dose of 1,000 mg/kg body weight/day or the reference controls over the same period (for both of which statistical significance was not observed) and was therefore considered to be unrelated to LNT administration.

LNT administration had no effect on pre-weaning development [as evaluated by the age of attainment of the surface and air righting reflexes, and the pupil reflex and startle response tests conducted on Day 14 of treatment (Day 20 of age)]. Ulna length and growth were similar between LNT-treated groups and controls. No test item-related differences in behaviour of the animals during the in-hand and arena observations in Week 11 of treatment (Day 81 to 83 of age) were observed. Morris maze performance was also unaffected by administration of LNT, with clear evidence of learning and memory over the 4 days of testing, as demonstrated by generally progressive decreases in group mean trial times, sector entries, and failed trials.

There were no test item-related differences for the mean body weight or day of age at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation and vaginal opening for males and females, respectively). The mean body weight at attainment of sexual maturation for females given 2,500 or 4,000 mg LNT/kg body weight/day was statistically significantly higher than for vehicle controls. However, the values were similar to those of reference controls and the differences from vehicle controls were minor, with no dose-response relationship observed.

No test item-related differences in values for haematological parameters between LNT-treated groups and vehicle controls were observed. At the end of the treatment period, statistically significantly increased mean cell haemoglobin (for males given 4,000 mg LNT/kg body weight/day) and mean cell haemoglobin concentration (for both sexes given 4,000 mg LNT/kg body weight/day) compared with vehicle controls were observed, but the differences were minor and values were similar to those for reference controls. Statistically significant reductions in red cell distribution width (for females given 2,500 or 4,000 mg LNT/kg body weight/day), mean cell volume and haematocrit (for females given 4,000 mg LNT/kg body weight/day) were not associated with a dose response, similar changes were not seen for males and the values were similar to those seen for reference controls. The majority of individual values for all of these parameters were also within the respective historical control data (HCD) ranges.

A statistically significant increase in leukocytes for males dosed at 4,000 mg LNT/kg body weight/day was a result of increased lymphocytes, eosinophils, and large unstained cells (LUC), which was not observed for female groups (values for the female high-dose LNT group were lower or equal to those of vehicle controls for the same parameters). These differences were considered to be due to vehicle control males having atypically low leukocyte values, as evidenced by the historical control ranges for all of these parameters: 5 of the 10 vehicle control males had values below the HCD lower limit for animals of the same strain and age (8.77 x 10^9/L). The mean value for this group (8.70 x 10^9/L) was also notably lower than the HCD mean (14.21 x 10^9/L) and below the HCD lower limit for individuals. In comparison, only 4, 3, and 2 individual values for males given 1,000, 2,500, or 4,000 mg LNT/kg body weight/day were below the HCD lower limit and the mean value for high-dose males (12.67 x 10^9/L) was the nearest of any group to the HCD mean. Similarly for lymphocytes and LUC, 3 and 5 out of 10 individual male vehicle control values, respectively, were below the HCD lower limit, whereas for high-dose males, all individual values and 9 of the 10 LUC values were within the HCD ranges for these parameters; mean values for high-dose males (10.81 and 0.08 x 10^9/L for lymphocytes and LUC, respectively) were very similar to the respective HCD means (10.72 and 0.11 x 10^9/L for lymphocytes and LUC, respectively), as opposed to vehicle control means (7.13 and 0.05 x 10^9/L for lymphocytes and LUC, respectively), which were notably lower than the HCD values. Statistically significant changes among females were limited to a decreased neutrophil concentration at 4,000 mg LNT/kg body weight/day, which again was inconsistent between the sexes as it was not seen for males and all but one of the individual values for this group were within the HCD range. Further evidence that these differences were clearly not test-item related include the lack of any associated changes that would usually correlate with effects on immune function; for example, there were no differences in rectal temperature as assessed in the in-hand observations, no test item-related differences in spleen weights nor any macroscopic or microscopic findings related to LNT administration.

Platelets were statistically significantly increased for males given 4,000 mg LNT/kg body weight/day compared with vehicle controls, but there was no dose response relationship nor any consistency between the sexes.

There were also no biologically relevant differences in the other clotting parameters [prothrombin time (PT) and activated partial thromboplastin time (APTT)] for LNT-treated groups compared with controls.

No test item-related differences in values for blood chemistry parameters between LNT-treated groups and vehicle controls were observed. Where statistically significant differences compared with vehicle controls were observed, there was either no dose-response relationship or the differences were inconsistent between the sexes. For all parameters, high dose values were comparable with those of reference controls and individual values for all LNT-treated groups were generally within HCD ranges. In the absence of any test item related effects in the treatment period, minor statistically significant differences between LNT-treated groups and vehicle controls observed during the recovery period were considered biologically irrelevant and unrelated to LNT administration.

There were no test item-related differences in urinalysis parameters between LNT-treated groups and controls. For females, a statistically significant increase in urine volume (at 4,000 mg LNT/kg body weight/day) and statistically significantly reduced specific gravity for all LNT-treated female groups were observed at the end of the treatment period compared with vehicle controls. There were no statistically significant differences in urine volume for LNT-treated males, but specific gravity was statistically significantly lower than vehicle controls for males given 2,500 or 4,000 mg LNT/kg body weight/day. These findings were considered biologically irrelevant and unrelated to LNT administration as all individual values for these parameters were within the HCD ranges for animals of this age and strain, indicating the values were within normal biological variation; there was also no evidence of an alteration in kidney function; there were no biologically relevant differences in total protein (statistically significant differences for males were not associated with a dose response), creatinine, or glucose concentrations between LNT-treated groups and controls, nor were there any microscopic abnormalities in urine sediment; and no test item-related differences in associated blood chemistry parameters, kidney weights, or in the incidences of macroscopic or microscopic kidney findings were observed.

Organ weights were unaffected by LNT administration. The only statistically significant differences in body weight-relative organ weights observed in LNT-treated groups compared to vehicle controls at the end of the treatment period were increases in testes weights for males given 2,500 or 4,000 mg LNT/kg body weight/day, but there was no evidence of a dose response. Statistically significantly increased body weight-relative kidney, liver, ovary, and spleen weights for females given 4,000 mg LNT/kg body weight/day were only seen at the end of the treatment-free period and were not evident immediately after cessation of dosing, indicating that these changes were unrelated to treatment with LNT. As there was no effect on the pituitary-thyroid axis observed during the study, the samples collected for potential analysis of TSH, T3, and T4 were not analysed; this is in accordance with OECD Test Guideline 407 (OECD, 2008), which the EFSA *Guidance for submission for food additive evaluations* refers to regarding modification of OECD Test Guideline 408 (OECD, 1998b) studies, to include assessment of some additional parameters that place more emphasis on endocrine-related endpoints (EFSA, 2012b).

Macroscopic and microscopic findings at scheduled necropsy revealed only incidental findings in all groups that are commonly observed in Sprague-Dawley rats of this age.

In absence of any test item-related adverse effects, the NOAEL for LNT was concluded to be 4,000 mg/kg body weight/day (the highest dose tested and maximum feasible dose, limited by viscosity of the test article).

Genotoxicity Studies

Bacterial Reverse Mutation Test

The potential mutagenicity of LNT was evaluated in a bacterial reverse mutation test (Ames test) (Phipps *et al.*, 2018b; Šoltésová, 2018b [unpublished]). This study was performed in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 471 (OECD, 1997), Commission Regulation (EC) No 440/2008[12] B13/14, U.S. Environmental Protection Agency (EPA) Health Effects Test Guidelines OPPTS

[12] Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). OJ L 142, 31.5.2008, p. 1–739. Available at: https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32008R0440 (current consolidated version: 16/10/2019).

870.5100 (U.S. EPA, 1998) and U.S. FDA Redbook IV.C.1.a. (U.S. FDA, 2000).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were treated with LNT at concentrations of up to 5,106.1 µg/plate (due to a minor error in the application of the correction factor, the high concentration slightly exceeded the intended high concentration of 5,000 µg/plate — the OECD 471 guideline maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix).

Water (purified by reverse osmosis) served as the vehicle for LNT and as the negative control. Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide) and presence [2-aminoanthracene and benzo(a)pyrene] of metabolic activation. A positive result for mutagenicity was defined as a dose-dependent and biologically relevant 2- or 3-fold increase in the number of revertant colonies, compared to that of the vehicle control group.

There was no evidence of mutagenicity following exposure to LNT in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in revertant colony counts (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that LNT is non-mutagenic at concentrations up to 5,106.1 µg/plate (slightly above the regulatory maximum dose level).

<u>In Vitro Mammalian Cell Micronucleus Test</u>

The clastogenic and aneugenic potential of LNT was evaluated in an *in vitro* mammalian cell micronucleus test conducted using human lymphocytes (Gilby, 2018b [unpublished]; Phipps *et al*., 2018b). This study was done in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 487 (OECD, 2016).

An initial preliminary cytotoxicity test was conducted using LNT at concentrations up to 2,042.44 µg/mL[13], in the presence (3-hour treatment) and absence (3 and 24-hour treatments) of S9 metabolic activation; no cytotoxicity was observed at any dose level. Cytotoxicity was assessed again in the main experiment and again there was no evidence of cytotoxicity at any dose level under any of the experimental conditions.

In the main experiment for micronucleus analysis, human lymphocytes were treated with concentrations of LNT at 510.61, 1,021.22, or 2,042.44 µg/mL with S9 (3 hours) and without S9 (3- and 24-hour treatments). The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronucleated binucleated cells (MNBC), with the frequency of MNBC also being above upper historical vehicle control limit.

There was no evidence of clastogenicity or aneugenicity in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in MNBC (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that LNT is neither clastogenic nor aneugenic at concentrations up to 2,042.44 µg/mL, in the absence and presence of metabolic activation.

C.2.1.3 Studies Conducted with Glycom's 6'-SL Sodium Salt

The same test article (Batch CPN5315 1000317 FD), composed of 96.8 % 6'-SL on a dry weight basis, was evaluated in all studies

Repeated-Dose Studies

<u>14-Day Toxicity Study in the Neonatal Rat</u>

[13] Due to a minor error in the application of the correction factor, the high concentration slightly exceeded the intended high concentration of 2,000 µg/mL—the OECD 487 guideline maximum recommended concentration.

A 14-day repeat dose toxicity study was conducted in rats to evaluate the potential short-term toxicity of 6'-SL sodium salt and select dose levels for the subsequent 90-day study (Flaxmer, 2018c [unpublished]).

Groups of 8 male and 8 female neonatal rats were dosed with 0 (water for irrigation), 4,000, or 5,000 mg/kg body weight/day (doses expressed on a basis) of 6'-SL sodium salt, by oral gavage at a dose volume 10 mL/kg body weight, once daily for 14 days, until the day before necropsy. Doses of 6'-SL sodium salt were corrected to account for "other carbohydrates" within the test article batch.

All animals were observed daily for changes in clinical condition. Body weights were recorded daily until the end of the dosing period, when animals were subjected to a gross macroscopic necropsy.

There were no deaths and no test item-related clinical signs. On the first day of dosing (Day 7 of age) 4 pups [1 control female and 3 animals (1 male and 2 females) receiving 4,000 mg 6'-SL sodium salt/kg body weight/day] were noted to have slight dose reflux immediately after the dosing procedure. Also, on the first day of dosing, 1 male receiving 5,000 mg 6'-SL sodium salt/kg body weight/day gasped for approximately 10 seconds immediately after the dosing procedure. These observations were considered to be incidental and unrelated to the test item, as all of these animals were observed as normal at the "end of group" dose observation on that day and no further clinical observations were seen for the remainder of the study.

There were no test item-related differences in body weight between 6'-SL sodium salt-treated groups and controls. Group mean body weights for females given 4,000 mg 6'-SL sodium salt/kg body weight/day were 10 % higher than those of controls at the end of the treatment period. However, this was primarily due to females given 4,000 mg/kg body weight/day being 7 % heavier than controls on the first day of dosing. The overall body weight gain for females given 4,000 mg 6'-SL sodium salt/kg body weight/day was also slightly higher (11 %) than controls, but there was no evidence of a dose-response (females given 5,000 mg 6'-SL sodium salt/kg body weight/day were only 6 % heavier than controls at the end of the dosing period and had gained only 8 % more weight overall). Therefore, these differences were considered to be unrelated to the test item. No test item-related macroscopic abnormalities were observed.

In the absence of any test item-related adverse findings, 5,000 mg 6'-SL sodium salt/kg body weight/day (the maximum tolerated dose, based on data for similar compounds) was considered the no-observed-adverse-effect level (NOAEL) and a suitable high-dose for the 90-day study.

90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential sub-chronic toxicity of 6'-SL sodium salt when administered orally, by gavage, to neonatal rats from Day 7 of age (Flaxmer, 2018d [unpublished]; Phipps *et al*., 2019a). The study was conducted in compliance with the Organisation for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b) with an adaptation for the use of neonatal animals.

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 1,000, 3,000, or 5,000 mg 6'-SL sodium salt/kg body weight/day (doses expressed on a basis), by oral gavage at a dose volume of 10 mL/kg body weight, once daily for at least 90 days, until the day before necropsy. An additional reference Control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 5,000 mg/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose 6'-SL sodium salt group and identify any effects related to the general fibre-like characteristics of the reference material. Doses of 6'-SL sodium salt and the reference control were corrected to account for "other carbohydrates" within the test article batches. A further 5 males and 5 females in each group were also dosed once daily for at least 90 days and then kept un-dosed for 4 weeks, to assess the reversibility of any observed effects.

Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of treatment until weaning and twice weekly thereafter. Food intake was recorded twice weekly from weaning until necropsy. The eyes of vehicle control, reference control and high-dose animals were examined in Week 13. Blood samples were taken for haematology, blood chemistry, and coagulation during Week 13 and at the end of the treatment-free period; urine samples were collected for urinalysis in Week 13 and at the end of the treatment-free period.

In Weeks 11 and 12, all animals were subjected to a functional observational battery consisting of observations in-hand and in a standard area, in addition to an assessment of grip strength and learning and memory (using

the Morris water maze). Pre-weaning reflex development (eye opening, air righting, startle response, and pupil closure response), ulna length, and sexual maturation (balano-preputial separation and vaginal opening) were also recorded for all animals during the treatment period.

At the end of the treatment and treatment-free periods, all surviving animals were subjected to a gross macroscopic necropsy, where (for all animals after the dosing period and for vehicle control, reference control, and high-dose 6'-SL sodium salt animals only, after the recovery period) selected organs (adrenal glands, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary gland, prostate, submandibular and sublingual salivary glands, seminal vesicles, spleen, testes, thymus, thyroid/parathyroid glands, and uterus/cervix) were weighed and fixed. At the end of the treatment period, a full list of tissues [adrenal glands, aorta, brain, caecum, colon, duodenum, epididymides, eyes, femur, Harderian glands, head, heart, ileum, jejunum, kidneys, liver, lungs, mesenteric and left axillary lymph nodes, oesophagus, ovaries, pancreas, pituitary gland, prostate, salivary glands, sciatic nerves, seminal vesicles, skeletal muscle, skin (with mammary glands), spinal cord, spleen, sternum, stomach, testes, thymus, thyroid glands (with parathyroids), trachea, urinary bladder, uterus (with cervix), and vagina] for early decedents and animals in the vehicle control and high-dose 6'-SL sodium salt groups, were examined microscopically. The testes and epididymides were examined microscopically at the end of the treatment period (for all male groups) and at the end of the treatment-free period (for vehicle control, reference control and high-dose males).

There were no test item-related deaths, clinical signs, or ocular changes. One reference control male was euthanised on Day 88 of dosing after showing clinical signs including gasping and unresponsiveness. The only notable macroscopic findings were depressions on the kidneys, which correlated with a minimal severity infiltrate of mononuclear inflammatory cells in the renal cortex seen microscopically. There were no other notable microscopic findings and the cause of the animal's poor clinical condition could not be identified, but as it was an isolated instance it was considered unrelated to administration of the reference control. One male given 5,000 mg 6'-SL sodium salt/kg body weight/day was found dead on Day 20 of dosing, with no notable macroscopic or microscopic findings reported. As it was an isolated instance it was considered unrelated to administration of 6'-SL sodium salt.

No biologically relevant differences in body weight or food consumption between 6'-SL sodium salt-treated groups and controls were observed. Statistically significant reductions in overall food consumption for males and females given 5,000 mg 6'-SL sodium salt/kg body weight/day were not associated with a dose-response and the differences from vehicle controls were minor (-1 g/animal/day).

Administration of 6'-SL sodium salt had no effect on pre-weaning development (as evaluated by the age of attainment of the surface and air righting reflexes, and the pupil reflex and startle response tests conducted on Day 14 of dosing). No test item-related effects on ulna length or growth were observed [statistically significantly increased overall mean ulna growth for 6'-SL sodium salt-treated male groups was clearly unrelated to the test item, as there was no dose-response relationship, the differences from vehicle controls were minor (≤ 1.4 mm) and there were no differences observed for females]. Behaviour of the animals during the in-hand and arena observations in Week 11 of dosing was unaffected by 6'-SL sodium salt. Morris maze performance was also unaffected by administration of 6'-SL sodium salt, with clear evidence of learning and memory over the 4 days of testing, as demonstrated by generally progressive decreases in group mean trial times, sector entries and failed trials.

There were no test item-related differences among the groups for the mean body weight or day of age at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation and vaginal opening, respectively). Where statistically significant differences were observed, they were not associated with a dose-response (longer time of completion for balano preputial separation for males given 1,000 mg 6'-SL sodium salt/kg body weight/day, shorter time of completion for vaginal opening for females given 3,000 mg 6'-SL sodium salt/kg body weight/day, lower body weight at time of vaginal opening for all 6'-SL sodium salt-treated female groups).

Administration of 6'-SL sodium salt had no effect on haematology and coagulation parameters. At the end of the treatment period, prothrombin time was statistically significantly shorter for males given 3,000 or 5,000 mg 6'-SL sodium salt/kg body weight/day and females given 5,000 mg 6'-SL sodium salt/kg body weight/day, compared with vehicle controls. Most of the individual values for 6'-SL sodium salt-treated males were within the historical control data (HCD) range (2 vehicle control male values were outside the HCD range, compared to only 1 of the individual male values at 5,000 mg 6'-SL sodium salt/kg body weight/day), which indicates that values were generally within normal biological variation. For females, there was no dose-

response relationship. The high-dose 6'-SL sodium salt male and female mean values (20.4 and 19.2 seconds, respectively) were also both comparable with the respective values for reference control males and females (20.5 and 19.8 seconds, respectively). Other statistically significant differences were also unrelated to the test item, as they were not associated with a dose-response [reduced haemoglobin for males in all 6'-SL sodium salt groups and females given 5,000 mg 6'-SL sodium salt/kg body weight/day; reduced haematocrit and red blood cells (RBC) for females given 5,000 mg 6'-SL sodium salt/kg body weight/day; lengthened activated partial thromboplastin time (APTT) for females given 5,000 mg 6'-SL sodium salt/kg body weight/day; reduced platelets for all female 6'-SL sodium salt groups; increased eosinophils for females given 5,000 mg 6'-SL sodium salt/kg body weight/day].

There were no test item-related effects on blood chemistry parameters. At the end of the treatment period, chloride was statistically significantly reduced for males and females receiving 3,000 or 5,000 mg 6'-SL sodium salt/kg body weight/day. In the absence of any gastrointestinal fluid loss due to diarrhoea in this study (which could cause a reduction in chloride values), a genuine test item-related adverse effect on chloride values would be associated with some evidence of an alteration in kidney function, but again this was not observed (there were no biologically relevant differences in creatinine or glucose concentrations between 6'-SL sodium salt-treated groups and controls, no microscopic abnormalities in urine sediment and no test item-related- differences in kidney weights nor any test item-related macroscopic or microscopic kidney findings observed). Where other statistically significant findings were observed, they were not associated with a dose-response [increased aspartate transaminase (AST) for all male 6'-SL sodium salt groups; increased albumin/globulin (A/G) ratio for males given 3,000 or 5,000 mg 6'-SL sodium salt/kg body weight/day; reduced total protein for all male and female 6'-SL sodium salt groups; reduced bilirubin for males given 1,000 or 3,000 mg 6'-SL sodium salt/kg body weight/day; reduced cholesterol for males and females given 5,000 mg/kg body weight/day; reduced albumin for all female 6'-SL sodium salt groups] and/or were inconsistent between the sexes (reduced potassium for males given 5,000 mg 6'-SL sodium salt/kg body weight/day and reduced sodium for females given 3,000 or 5,000 mg 6'-SL sodium salt/kg body weight/day).

No test item-related or biologically relevant differences in urinalysis parameters between 6'-SL sodium salt-treated groups and controls were observed. Total urinary protein was statistically significantly reduced for male and female animals receiving 5,000 mg 6'-SL sodium salt/kg body weight/day, but for males there was no dose-response. Furthermore, all individual male values were within the HCD range and the same number of individual female vehicle control values (2) were outside the HCD range as in the 5,000 mg 6'-SL sodium salt/kg body weight/day group, indicating that high-dose values reflected normal biological variation.

There were no test item-related differences in organ weights between 6'-SL sodium salt-treated groups and vehicle controls at the end of the dosing or recovery periods. Where statistically significant differences were observed, the differences were either not associated with a dose-response (reduced body weight adjusted salivary glands for females receiving 3,000 or 5,000 mg 6'-SL sodium salt/kg body weight/day) or were only seen in 1 sex (increased body weight adjusted liver weights for males receiving 3,000 or 5,000 mg 6'-SL sodium salt/kg body weight/day; reduced body weight adjusted heart weights for females receiving 3,000 or 5,000 mg 6'-SL sodium salt/kg body weight/day). As there was no effect on the pituitary-thyroid axis observed during the study, the samples collected for potential analysis of thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) were not analysed; this is in accordance with OECD Test Guideline 407 (OECD, 2008), which the EFSA *Guidance for submission for food additive evaluations* refers to regarding modification of OECD Test Guideline 408 (OECD, 1998b) studies, to include assessment of some additional parameters that place more emphasis on endocrine-related endpoints (EFSA, 2021).

There were no biologically relevant macroscopic or microscopic findings at 1,000 or 3,000 mg 6'-SL sodium salt/kg body weight/day. Four males given 5,000 mg 6'-SL sodium salt/kg body weight/day had unilateral tubular atrophy in the testis and absence of sperm in the epididymis on the same side. Complete unilateral testicular tubular atrophy is occasionally seen in young male rats as an incidental background change (McInnes, 2012; Sahota *et al.*, 2017) and given that it was seen unilaterally with spermatogenesis in the contralateral testis unaffected, it is highly unlikely that there was any direct effect of 6'-SL sodium salt on spermatogenesis, where a bilateral change would be expected. Additionally, a genuine test item-related change would likely affect at least a small number of animals in the lower dose groups at a milder severity, but there were no similar findings (of any severity) in the low- or mid-dose groups. In all cases in this study, the severity of the atrophy in the testis and absence of sperm in the epididymis indicate a level of chronicity rather than an ongoing degenerative change, possibly even of a condition pre-existing before start of study. The absence of these findings in the recovery animals substantiates this, as complete recovery of these testicular

and epididymal findings would not be expected after only 4 weeks. There were also no test item-related macroscopic or microscopic findings reported in another 90-day toxicity study conducted with 6'-SL sodium salt (Gurung *et al*., 2018), which further supports the conclusion that these findings were highly likely to be incidental and unrelated to administration of 6'-SL sodium salt. Although unilateral tubular atrophy can be observed as a consequence of blockage of the excurrent duct system (Sahota *et al.*, 2017), there are several features that are inconsistent with this mechanism in this case. With excurrent ductular blockage there are often accompanying changes, such as increased testicular weight due to fluid accumulation, chronic inflammatory or atrophic changes of the affected segment of the epididymis, rete tubular dilatation or dilation of the testicular and/or epididymal tubules due to stasis and fluid/sperm accumulation. However, none of these changes were evident in any of the 4 animals with the testicular/epididymal findings in this study and thus, unilateral atrophy secondary to blockage seems highly unlikely to be the pathogenesis for these findings.

Unilateral tubular atrophy (testis) and absent sperm (epididymis) for 4 males given 5,000 mg/kg body weight/day were highly unlikely to be test item-related and no scientifically plausible explanation can be given for these effects, but the incidences of these findings were outside the historical control range for studies of this type.

Therefore, in the absence of any test item-related adverse findings at lower doses, 3,000 mg 6'-SL sodium salt/kg body weight/day (total carbohydrate amount of 3,132 mg/kg body weight/day) was considered to represent a conservative NOAEL. It is noted that this NOAEL was also supported by the EFSA NDA Panel in the Scientific Opinion (EFSA, 2020a).

Genotoxicity Studies

Bacterial Reverse Mutation Test

The potential mutagenicity of 6'-SL sodium salt was evaluated in a bacterial reverse mutation test (Ames test) (Šoltésová, 2018c [unpublished]; Phipps *et al*., 2019a). This study was performed in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 471 (OECD, 1997), Commission Regulation (EC) No 440/2008[14] B13/14, U.S. Environmental Protection Agency (EPA) Health Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998) and U.S. FDA Redbook IV.C.1.a. (U.S. FDA, 2000).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were exposed to 6'-SL sodium salt at concentrations of up to 5,000 µg/plate (the OECD Test Guideline 471 maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix) (OECD, 1997). Doses of 6'-SL sodium salt were corrected to account for “other carbohydrates” within the test article batch

Water (purified by reverse osmosis) served as the vehicle for 6'-SL sodium salt and as the negative control. Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene and 4-nitroquinoline-1-oxide) and presence (2-aminoanthracene and benzo[a]pyrene) of metabolic activation. A positive result for mutagenicity was defined as a dose-dependent and biologically relevant 2- or 3-fold increase in the number of revertant colonies, compared to that of the vehicle control group.

There was no evidence of mutagenicity in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced increases in mean revertant colony numbers of at least twice (or three times in the case of strains TA1535 and TA1537) that of the concurrent vehicle controls (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that 6'-SL sodium salt is non-mutagenic at concentrations up to 5,000 µg/plate

[14] Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). OJ L 142, 31.5.2008, p. 1–739. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex:32008R0440 (Consolidated Version: 16/10/2019).

(the OECD Test Guideline 471 maximum recommended concentration) (OECD, 1997).

In Vitro Mammalian Cell Micronucleus Test

The clastogenic and aneugenic potential of 6'-SL sodium salt was evaluated in an *in vitro* mammalian cell micronucleus test, conducted using human lymphocytes (Gilby, 2018c [unpublished]; Phipps *et al*., 2019a). This study was done in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 487 (OECD, 2016).

An initial preliminary cytotoxicity test was conducted using 6'-SL sodium salt at concentrations up to 2,000 µg/mL (the OECD Test Guideline 487 maximum recommended concentration), in the presence (3-hour treatment) and absence (3- and 24-hour treatments) of S9 metabolic activation; there was no evidence of cytotoxicity observed at any dose level (OECD, 2016). Cytotoxicity was assessed again in the main experiment, where there was no evidence of cytotoxicity at any dose level under any of the experimental conditions.

In the main experiment for micronucleus analysis, human lymphocytes were exposed to concentrations of 6'-SL sodium salt at 500, 1,000, or 2,000 µg/mL with S9 (3 hours) and without S9 (3- and 24-hour treatments) (concentrations expressed on a 6'-SL basis). Doses of 6'-SL sodium salt were corrected to account for "other carbohydrates" within the test article batch. The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronucleated binucleated cells (MNBCs) compared with vehicle controls, with the frequency of MNBCs also being above upper historical vehicle control limit.

There was no evidence of clastogenicity or aneugenicity in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in MNBC (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that 6'-SL sodium salt is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL (the OECD Test Guideline 487 maximum recommended concentration), in the absence and presence of metabolic activation (OECD, 2016).

C.2.1.4 Studies Conducted with Glycom's 3'-SL Sodium Salt

The same test article (Batch CPN5115 1000516 FD), composed of 90.3 % 3'-SL on a dry weight basis, was evaluated in all studies.

Repeated-Dose Studies

14-Day Toxicity Study in the Neonatal Rat

A 14-day repeat dose toxicity study was conducted in rats to evaluate the potential short-term toxicity of 3'-SL sodium salt and select dose levels for a subsequent 90-day study (Stannard, 2019a [unpublished]).

Groups of 8 male and 8 female neonatal Crl:CD(SD) rats were dosed with 0 (water for irrigation), 4,000, or 5,000 mg 3'-SL sodium salt/kg body weight/day, by oral gavage at a dose volume 10 mL/kg body weight, once daily for 14 days, until the day before necropsy. Doses of 3'-SL sodium salt were corrected to account for "other carbohydrates" within the test article batch.

All animals were observed daily for changes in clinical condition. Body weights were recorded daily until the end of the dosing period, when animals were subjected to a gross macroscopic necropsy.

There were no test item-related deaths or clinical signs. One male receiving 4,000 mg 3'-SL sodium salt/kg body weight/day was found dead at the final observation occasion on Day 14 of dosing. This animal had shown no changes in clinical condition but gained slightly less weight (2%) than the other males in this group (8 to 11%) between Days 13 and 14 of dosing. Macroscopic examination revealed no abnormalities and there was no evidence of dosing trauma. In the absence of any other deaths during the study, this premature death was considered incidental and unrelated to administration of 3'-SL sodium salt. There were no biologically relevant differences in body weight between test item-treated groups and controls and no test item-related macroscopic abnormalities at necropsy.

In the absence of any test item-related adverse findings, 5,000 mg 3'-SL sodium salt/kg body weight/day (the maximum tolerated dose, based on data for similar compounds) was considered the no-observed-adverse-effect level (NOAEL) and a suitable high dose for the 90-day study.

90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential sub-chronic toxicity of 3'-SL sodium salt when administered orally, by gavage, to neonatal Crl:CD(SD) rats from Day 7 of age (Phipps *et al.*, 2019b; Stannard, 2019b [unpublished]). The study was conducted in compliance with the Organisation for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) (OECD, 1998a) and according to OECD Test Guideline 408 (OECD, 1998b) with an adaptation for the use of neonatal animals.

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (water for irrigation), 1,000, 3,000, or 5,000 mg 3'-SL sodium salt/kg body weight/day, by oral gavage at a dose volume of 10 mL/kg body weight, once daily for at least 90 days, until the day before necropsy. An additional reference control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 5,000 mg/kg body weight/day under the same conditions, to allow for direct comparison against the high-dose 3'-SL sodium salt group and identify any effects related to the general fiber-like characteristics of the reference material. Doses of 3'-SL sodium salt and the reference control were corrected to account for “other carbohydrates” within the test article batches. A further 5 males and 5 females in each group were also dosed once daily for at least 90 days and then kept undosed for 4 weeks, to assess the reversibility of any observed effects.

Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of treatment until weaning and twice weekly thereafter. Food intake was recorded twice weekly from weaning until necropsy. The eyes of vehicle control, reference control and high-dose animals were examined in Week 13. Blood samples were taken for haematology, blood chemistry and coagulation during Week 13 and for blood chemistry only at the end of the treatment-free period. Urine samples were collected for urinalysis in Week 13 and at the end of the treatment-free period; water consumption was recorded 1 week before urine collection on each occasion.

In Weeks 11 and 12, all animals were subjected to a functional observational battery consisting of observations in-hand and in a standard area, in addition to an assessment of grip strength and learning and memory (using the Morris water maze). Pre-weaning reflex development (eye opening, air righting, startle response, and pupil closure response), ulna length, and sexual maturation (balano-preputial separation and vaginal opening) were also recorded for all animals during the treatment period.

At the end of the treatment and treatment-free periods, all surviving animals were subjected to a gross macroscopic necropsy, where (for all animals after the dosing period and for vehicle control, reference control, and high-dose 3'-SL sodium salt animals only, after the recovery period) selected organs (adrenal glands, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pituitary gland, prostate, submandibular and sublingual salivary glands, seminal vesicles, spleen, testes, thymus, thyroid/parathyroid glands, and uterus/cervix) were weighed and fixed. At the end of the treatment period, a full list of tissues [adrenal glands, aorta, brain, caecum, colon, duodenum, epididymides, eyes, femur, Harderian glands, head, heart, ileum, jejunum, kidneys, liver, lungs, mesenteric and left axillary lymph nodes, oesophagus, ovaries, pancreas, pituitary gland, prostate, salivary glands, sciatic nerves, seminal vesicles, skeletal muscle, skin (with mammary glands), spinal cord, spleen, sternum, stomach, testes, thymus, thyroid glands (with parathyroids), trachea, urinary bladder, uterus (with cervix), and vagina] for early decedents and animals in the vehicle control and high-dose 3'-SL sodium salt groups, were examined microscopically.

There were no test item-related deaths, clinical signs, or ocular changes. One low-dose female was euthanised on Day 74 of dosing, due to clinical signs of rapid respiration, thin build, and whole-body pallor; this female also lost weight (35 g) during the preceding 3 days. Macroscopic findings for this animal included thin, clear fluid in the thoracic and abdominal cavities, an enlarged heart, a firm liver and lungs and dark discolouration of several tissues. Histopathology revealed marked, haemorrhagic necrosis of the adrenal cortex (considered to be the major factor contributing to death), haemorrhagic necrosis in the centrilobular area of the liver, inflammatory infiltrate in the liver, marked thymic necrosis and a minor increase in haematopoiesis in the spleen. This isolated death was incidental and unrelated to administration of 3'-SL sodium salt.

No biologically relevant differences in body weight or food consumption between 3'-SL sodium salt-treated groups and controls were observed. Statistically significantly lower mean final body weight and overall mean body weight gain for males given 5,000 mg 3'-SL sodium salt/kg body weight/day, compared with vehicle controls, were considered to be unrelated to 3'-SL sodium salt, as the differences were minor (7%) and there was clearly no evidence of a dose-response. Furthermore, the mean final body weight and overall body weight gain values for the males given 5,000 mg 3'-SL sodium salt/kg body weight/day were almost identical (within < 1%) to those for reference controls. Males and females given 5,000 mg 3'-SL sodium salt/kg body weight/day drank slightly more water than vehicle controls during the 1-week recording period prior to urine collection in Week 13, but there was no dose-response relationship.

Administration of 3'-SL sodium salt had no effect on pre-weaning development (as evaluated by the age of attainment of the surface and air righting reflexes, and the pupil reflex and startle response tests conducted on Day 14 of dosing) or on ulna length or growth. Behaviour of the animals during the in-hand and arena observations in Week 11 of dosing was unaffected by 3'-SL sodium salt; forelimb grip strength and rearing counts were statistically significantly lower for females given 5,000 mg 3'-SL sodium salt/kg body weight/day compared with vehicle controls, but there was no dose-response for either parameter, and similar differences were not seen for males. The mean rearing count for low-dose females was largely affected by two atypically low individual values (5 rearings each) in this group (the other females in this group reared 10 to 23 times and vehicle controls reared 10 to 27 times). Morris maze performance was also unaffected by administration of 3'-SL sodium salt, with clear evidence of learning and memory over the 4 days of testing, as demonstrated by generally progressive decreases in group mean trial times, sector entries and failed trials.

There were no test item-related differences among the groups for the mean body weight or day of age at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation and vaginal opening, respectively).

No test item-related differences in values for haematological parameters between 3'-SL sodium salt-treated groups and vehicle controls were observed at the end of the treatment period. Statistically significantly decreased haemoglobin (for low-dose males and for females given 5,000 mg 3'-SL sodium salt/kg body weight/day) and red blood cell count (for females given 5,000 mg 3'-SL sodium salt/kg body weight/day) compared with vehicle controls were observed at the end of the dosing period, but there was no evidence of a dose-response and the differences were minor; high-dose values were also similar to those for reference controls. All individual values for these groups were within respective historical control ranges for these parameters.

Neutrophil concentrations for all groups of females given 3'-SL sodium salt were statistically significantly higher than vehicle control values; however, there was no dose-response, this difference was not seen for males and all individual values were within the historical control data (HCD) range. Prothrombin time was statistically significantly shorter for all groups of males and females given 3'-SL sodium salt, compared with vehicle controls. However, there was no dose-response for either sex and there were no corresponding differences in activated partial thromboplastin time (APTT) between test item-treated groups and controls. Furthermore, most of the individual values for the 3'-SL sodium salt groups were within HCD ranges, indicating that values were within normal biological variation. High-dose 3'-SL sodium salt values were also similar to those for reference controls.

There were no test item-related differences in values for blood chemistry parameters between 3'-SL sodium salt treated groups and vehicle controls at the end of the treatment or recovery periods. Statistically significant reductions in sodium (for all male 3'-SL sodium salt groups and females given 5,000 mg 3'-SL sodium salt/kg body weight/day), total protein (males given 5,000 mg 3'-SL sodium salt/kg body weight/day) and albumin (males given 5,000 mg 3'-SL sodium salt/kg body weight/day), in addition to statistically significantly increased albumin/globulin (A/G) ratio (for all male 3'-SL sodium salt groups) and creatinine (for males given 3,000 or

5,000 mg 3'-SL sodium salt/kg body weight/day) were clearly not test item-related, as there was no dose-response relationship and/or no consistency between the sexes, with all individual values being within respective HCD ranges, indicating that individual values reflect normal biological variation.

Statistically significant differences in triglycerides and urea (for both sexes given 5,000 mg 3'-SL sodium salt/kg body weight/day) and in sodium and chloride (for males given 3,000 or 5,000 mg 3'-SL sodium salt/kg body weight/day and females given 5,000 mg 3'-SL sodium salt/kg body weight/day) were also considered to be unrelated to 3'-SL sodium salt administration, as all individual values were within respective HCD ranges for these parameters, therefore reflecting normal biological variation rather than any effect of the test item. At the end of the treatment-free period, statistically significant differences in parameters for which differences were not observed at the end of the treatment period, were considered biologically irrelevant and unrelated to 3'-SL sodium salt administration. The only statistically significant difference observed at the end of the treatment-free period (increased A/G ratio for males given 5,000 mg 3'-SL sodium salt/kg body weight/day), was also unrelated to the test item, as the majority (4/5) of the individual values were within the HCD range for this parameter, reflecting normal biological variation.

No test item-related or biologically relevant differences in urinalysis parameters between 3'-SL sodium salt-treated groups and controls were observed. Statistically significantly decreased urine volume, total protein, and total creatinine, in addition to increased specific gravity, were observed for males given 5,000 mg 3'-SL sodium salt/kg body weight/day compared with vehicle controls, but there was no evidence of a dose-response for any of the parameters and these differences were not seen for females. Urinary pH was statistically significantly increased for all 3'-SL sodium salt-treated groups compared to vehicle controls, but individual values for this and the other urinary parameters were within the HCD ranges for animals of this age and strain, indicating the values were within normal biological variation. These findings were also considered biologically irrelevant and unrelated to 3'-SL sodium salt administration as there was also no evidence of an alteration in kidney function, no microscopic abnormalities in urine sediment, no test item-related differences in kidney weights and no test item-related macroscopic or microscopic kidney findings observed.

There were no test item-related differences in organ weights between 3'-SL sodium salt-treated groups and vehicle controls at the end of the dosing or recovery periods. At the end of the treatment period, statistically significantly decreased body weight-adjusted brain, testes and prostate weights for males given 5,000 mg 3'-SL sodium salt/kg body weight/day and increased adjusted kidney weights in all groups of 3'-SL sodium salt-treated females, compared with vehicle controls, were not associated with a dose-response. Mean adjusted salivary gland weight for males given 5,000 mg 3'-SL sodium salt/kg body weight/day was statistically significantly lower that for vehicle controls; this was considered to be a consequence of the non-dose related marginally lower absolute terminal body weight of the high-dose 3'-SL sodium salt males, given that there was no statistically significant difference in absolute weight. No statistically significant differences between test item-treated groups and vehicle controls were observed at the end of the treatment-free period. As there was no effect on the pituitary-thyroid axis observed during the study, the samples collected for potential analysis of thyroid stimulating hormone (TSH), triiodothyronine (T3), and thyroxine (T4) were not analysed; this is in accordance with OECD Test Guideline 407 (OECD, 2008), which the EFSA *Guidance for submission for food additive evaluations* refers to regarding modification of OECD Test Guideline 408 (OECD, 1998b) studies, to include assessment of some additional parameters that place more emphasis on endocrine-related endpoints (EFSA, 2012b).

Macroscopic and microscopic findings at scheduled necropsy revealed only incidental findings in all groups that are commonly observed in Sprague Dawley rats of this age.

In the absence of any test item-related adverse effects, the NOAEL for 3'-SL sodium salt was concluded to be 5,000 mg/kg body weight/day, the highest dose tested and maximum tolerated dose, based on data for similar compounds.

Genotoxicity Studies

Bacterial Reverse Mutation Test

The potential mutagenicity of 3'-SL sodium salt was evaluated in a bacterial reverse mutation test (Ames test), which was performed in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD

Test Guideline 471 (OECD, 1997), Commission Regulation (EC) No 440/2008[15] B13/14, U.S. Environmental Protection Agency (EPA) Health Effects Test Guidelines OPPTS 870.5100 (U.S. EPA, 1998) and U.S. FDA Redbook IV.C.1.a. (U.S. FDA, 2000) (Phipps *et al*., 2019b; Šoltésová, 2019 [unpublished]).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were exposed to 3'-SL sodium salt at concentrations of up to 5,000 µg 3'-SL sodium salt/plate (the OECD Test Guideline 471 maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix) (OECD, 1997). Doses of 3'-SL sodium salt were corrected to account for "other carbohydrates" within the test article batch.

Water (purified by reverse osmosis) served as the vehicle for 3'-SL sodium salt and as the negative control. Positive controls were also included in the absence (sodium azide, 9-aminoacridine, 2-nitrofluorene and 4nitroquinoline-1-oxide) and presence (2-aminoanthracene and benzo[a]pyrene) of metabolic activation. A positive result for mutagenicity was defined as a dose-dependent and biologically relevant 2- or 3-fold increase in the number of revertant colonies, compared to that of the vehicle control group.

There was no evidence of mutagenicity in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced increases in mean revertant colony numbers of at least twice (or three times in the case of strains TA1535 and TA1537) that of the concurrent vehicle controls (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

It was concluded, therefore, that 3'-SL sodium salt is non-mutagenic at concentrations up to 5,000 µg/plate (the OECD Test Guideline 471 maximum recommended concentration) (OECD, 1997).

In Vitro Mammalian Cell Micronucleus Test

The clastogenic and aneugenic potential of 3'-SL sodium salt was evaluated in an *in vitro* mammalian cell micronucleus test, conducted using human lymphocytes, in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Test Guideline 487 (OECD, 2016) (Gilby, 2019 [unpublished]; Phipps *et al*., 2019b).

An initial preliminary cytotoxicity test was conducted using 3'-SL sodium salt at concentrations up to 2,000 µg/mL (the OECD Test Guideline 487 maximum recommended concentration), in the presence (3-hour treatment) and absence (3- and 24-hour treatments) of S9 metabolic activation; there was no evidence of cytotoxicity observed at any dose level (OECD, 2016). Cytotoxicity was assessed again in the main experiment, where there was no biologically relevant indication of cytotoxicity at any dose level under any of the experimental conditions.

In the main experiment for micronucleus analysis, human lymphocytes were treated with concentrations of 250, 500, 1,000, or 2,000 µg 3'-SL sodium salt/mL with S9 (3 hours) and without S9 (3- and 24-hour treatments). Doses of 3'-SL sodium salt and the reference control were corrected to account for "other carbohydrates" within the test article batch. The vehicle (water, purified by reverse osmosis) was used as a negative control and positive controls were also included in the absence (colchicine and mitomycin C) and presence (cyclophosphamide) of metabolic activation. A positive result for clastogenicity/aneugenicity was defined as a dose-dependent, statistically significant increase in the frequency of micronucleated binucleated cells (MNBCs) compared with vehicle controls, with the frequency of MNBCs also being above upper historical vehicle control limit.

There was no evidence of clastogenicity or aneugenicity in any of the tests, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in MNBC (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations.

[15] Council Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). OJ L 142, 31.5.2008, p. 1–739. Available at: https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX%3A32008R0440 (current consolidated version: 16/10/2019).

It was concluded, therefore, that 3'-SL sodium salt is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL (the OECD Test Guideline 487 maximum recommended concentration), in the absence and presence of metabolic activation (OECD, 2016).

C.2.1.5 Summary

The safety of Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by fermentation is supported by a series of product-specific toxicological studies. A summary of the repeated-dose studies that have been conducted with Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt is presented in Table C.2.1.5-1 below, while the results of the genotoxicity/mutagenicity assays are summarised in Table C.2.1.5-2.

The safety of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt is further supported by toxicological studies that have been conducted with other preparations containing these HiMOs (including mixtures). Consistent with the expected safety of HiMOs that is established from their history of consumption in human milk, the results of the toxicity studies that have been conducted to date demonstrate these ingredients do not pose any toxicological concerns.

Table C.2.1.5-1 Summary of the Repeated-Dose Toxicology Studies Conducted with Glycom's 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3'-SL Sodium Salt Preparations

Test Article	Species	Route and Dose (mg/kg bw/day)	Duration	NOAEL (mg/kg bw/day)	Reference
Glycom's 2'-FL/DFL	Rats (Crl:CD(SD))	0, 4,000, or 5,000	14 days (DRF)	5,000	Flaxmer (2018a [unpublished])
	Rats (Crl:CD(SD))	0, 1,000, 3,000, or 5,000	90	5,000	Flaxmer (2018b [unpublished]); Phipps *et al.* (2018a)
Glycom's LNT	Rats (Crl:CD(SD))	Gavage; 0, 3,250 or 4,000	14 days (DRF)	4,000	Stannard, 2019a [unpublished]); Phipps *et al.* (2018b)
	Rats (Crl:CD(SD))	Gavage; 1,000, 2,500, or 4,000	90 days	4,000	Stannard, 2018 [unpublished]); Phipps *et al.* (2018b)
Glycom's 6'-SL sodium salt	Rats (Crl:CD(SD))	Gavage; 0, 4,000, or 5,000 (6'-SL basis)	14 days (DRF)	5,000 (6'-SL)	Flaxmer (2018c [unpublished])
	Rats (Crl:CD(SD))	Gavage; 0, 1,000, 3,000, or 5,000 (6'-SL basis)	90 days	3,000 (6'-SL)	Flaxmer (2018d [unpublished]); Phipps *et al.* (2019a)
Glycom's 3'-SL sodium salt	Rats (Crl:CD(SD))	Gavage; 0, 4,000, or 5,000 (3'-SL basis)	14 days (DRF)	5,000 (3'-SL)	Stannard (2019a [unpublished])
	Rats (Crl:CD(SD))	Gavage; 0, 1,000, 3,000, or 5,000 (3'-SL basis)	90 days	5,000 (3'-SL)	Stannard (2019b [unpublished]); Phipps *et al.* (2019b)

2'-FL/DFL = 2'-fucosyllactose/difucosyllactose mixture; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; bw = body weight; DRF = dose-range finding study; LNT = lacto-*N*-tetraose; NOAEL = no-observed-adverse-effect level.

Table C.2.1.5-2 Summary of the Genotoxicity Studies Conducted with Glycom's 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3'-SL Sodium Salt Preparations

Test Article	Assay	Concentrations	Metabolic Activation	Result	Reference
Glycom's 2'-FL/DFL	Bacterial reverse mutation assay	Up to 5,000 µg/plate	± S9	Negative	Phipps *et al.* (2018a); Šoltésová (2018a [unpublished])
	In vitro mammalian cell micronucleus assay	500, 1,000, or 2,000 µg/mL	± S9	Negative	Gilby (2018a [unpublished]); Phipps *et al.* (2018a)
Glycom's LNT	Bacterial reverse mutation assay	5.1, 15.3, 51.1, 153.2, 510.6, 1531.8, or 5106.1 µg/plate	± S9	Negative	Phipps *et al.* (2018b); Šoltésová (2018b [unpublished])
	In vitro mammalian cell micronucleus assay	511, 1,021, or 2,042 µg/mL	± S9	Negative	Gilby (2018b [unpublished]); Phipps *et al.* (2018b)
Glycom's 6'-SL sodium salt	Bacterial reverse mutation assay	Up to 5,000 µg/plate (6'-SL basis)	± S9	Negative	Phipps *et al.* (2019a); Šoltésová (2018c [unpublished])
	In vitro mammalian cell micronucleus assay	500, 1,000 or 2,000 µg/mL (6'-SL basis)	± S9	Negative	Gilby (2018c [unpublished]); Phipps *et al.* (2019a)
Glycom's 3'-SL sodium salt	Bacterial reverse mutation assay	Up to 5,000 µg/plate (3'-SL basis)	± S9	Negative	Phipps *et al.* (2019b); Šoltésová (2019 [unpublished])
	In vitro mammalian cell micronucleus assay	Up to 2,000 µg/plate (3'-SL basis)	± S9	Negative	Gilby (2019 [unpublished]); Phipps *et al.* (2019b)

2'-FL/DFL = 2'-fucosyllactose/difucosyllactose mixture; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; LNT = lacto-*N*-tetraose.

C.2.2 Clinical Data

Three clinical studies have been conducted in infants using mixtures of 5 HiMOs which included 2'-FL, LNT, 6'-SL, and 3'-SL and either DFL (Cohen, 2022 [unpublished]) or 3-FL (Parschat *et al.*, 2021; Lasekan *et al.*, 2022). Cohen (2022, [unpublished]) specifically evaluated Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt. These studies demonstrate that the supplementation of infant formula with HiMOs at levels of up to 5.75 g/L, with levels of individual HiMOs within naturally occurring ranges in human milk (including those under evaluation, *i.e.*, 2'-FL, DFL, LNT, 6'-SL, and 3'-SL), is safe, well tolerated, and supported appropriate growth in infants. A detailed summary of the Parschat *et al.* (2021) and Lasekan *et al.* (2022) studies is presented below. For the Cohen (2022 [unpublished]) study, an abbreviated summary is presented below, while the full unpublished study report is provided in Appendix IX **(Confidential Commercial Information)**.

Tabular summaries of clinical studies not previously evaluated by FSANZ[16] conducted in infants administered infant formula supplemented with 2'-FL alone or in combination with LNnT (LNT's constitutional isomer) are provided in Annex 1.

Double-Blind, Randomised, Controlled, Growth and Safety Study of Formula for Infants and Young Children Containing 5 HiMO Blend (Cohen, 2022 [Unpublished])

The growth, safety, and efficacy of a bovine milk-based whey predominant term starter infant formula, follow-up formula, and growing up milk containing 5 HiMOs was investigated in a double-blind, randomised, controlled, multicentre trial[1] [Cohen, 2022 (unpublished); Clinical Trial Registry NCT 03722550]. Healthy term infants 7 to 21 days of age and exclusively consuming cow's milk infant formula were eligible for the study.

[16] For studies previously evaluated by FSANZ, see Applications A1155, A1190, and A1233.

Enrolled infants were randomised to the Control Group, Test Group 1, or Test Group 2. A non-randomised Reference Group of healthy term, exclusively breastfed infants was enrolled in parallel.

The age-specific formulas contained nutrients in amounts intended for full nutritional support of infants 0 to 6 months of age (starter infant formula), and for the complementary nutrition of infants 6 to 12 months of age (follow-up formula) and young children 12 to 15 months (growing-up milk). Study formulas were identical to the control formula except for the replacement of part of the lactose content with the HiMOs 2'-FL, DFL, LNT, 3'-SL, and 6'-SL. There were two Test Groups: infants in Test Group 1 received starter infant formula with 1.5 g/L total HMOs, while infants in Test Group 2 received starter infant formula with 2.5 g/L total HMOs. Infants from both test groups received follow-up formula with 0.5 g/L total HMOs from 6 to 12 months, and a growing up milk with 0.4 g/L total HMOs past 12 months. Levels of the individual HMOs were based on the profile naturally found in human milk. Complementary foods were allowed when the infant reached 4 months (120 days) of age.

The co-primary objectives of the trial were to demonstrate safety (weight gain from enrolment to 4 months of age), and efficacy (recurrent incidences of illness throughout the entire study) in the test formulas containing HiMOs compared to control formula. Secondary objectives of this trial were to compare additional safety and efficacy outcomes (including anthropometry, gastrointestinal tolerance, adverse events, medication use, faecal microbiome composition, faecal metabolic profile, and faecal markers of immune and gut health) between the Test, Control, and Reference Groups at 3, 6, 12, and 15 months of age. The co-primary endpoint of weight gain from enrolment to 4 months was evaluated in the full-analysis set (FAS)[17] and the per-protocol (PP)[18] populations. Total adverse events (including the co-primary endpoint of respiratory tract infections) were conducted within the safety analysis set[19]. Secondary endpoints were conducted in the FAS only.

From a total of 792 eligible infants screened, 693 infants were randomised to an intervention group, with an additional 96 exclusively breastfed infants allocated to the reference group; 3 infants did not meet the inclusion criteria. In the formula groups, 686 infants received the allocated intervention representing the full analysis set. By the 4-month visit, 109 infants from formula-fed groups had major protocol deviations, resulting in 577 formula-fed infants representing the per protocol group at this timepoint. A total of 133 infants withdrew from the study by the 12-month follow-up visit across all groups (Control: 40 infants; Test Group 1: 44 infants; Test Group 2: 38; breastfed Reference Group: 11 infants). Analyses on secondary outcomes (including faecal microbiota, pH, organic acids, and markers of intestinal immune response, permeability, and inflammation) were performed in 535 infants.

Safety Analyses (through to 4 months and 12 months of age)

Weight gain was assessed through to 4 months of age according to recommendations from the U.S. Food and Drug Administration and the American Academy of Paediatrics for clinically testing the safety of infant formula. The lower bound of the 95% confidence interval for the mean difference in daily body weight gain from enrolment through 4 months of age was above the non-inferiority margin of -3 g/day when comparing the two Test Groups with the Control Group (non-inferiority $p<0.001$ in FAS and PP populations). Gains in body weight, length, and head circumference from baseline through 12 months of age were comparable among the formula-fed groups and tracked with the World Health Organisation (WHO) median values through to 12 months of age. Heart rate, body temperature, and respiratory rate were generally also similar among formula-fed groups. Mean weight-for-age, length-for-age, head circumference-for-age, and BMI-for-age z-scores demonstrated comparable growth among all formula groups compared with WHO standard growth curves (largely within 0.5 standard deviations) through 12 months of age. Only the weight-for-length percentile was significantly greater in Test Group 2 compared to Control ($p<0.05$) at 9 months of age though both groups generally tracked well against the WHO standard growth curves (mean z-score within 1 standard deviation at this timepoint).

[17] All randomized subjects in the three treatment groups who received the allocated product as well as the breastfed reference group.
[18] All subjects in the FAS population who adhered to all protocol requirements without any major protocol violations and with complete baseline data and at least one post-randomization data point.
[19] All randomized infants with documented use of at least one feeding of the study formula.

Measures of gastrointestinal tolerance and associated behaviours, including stool frequency, difficulty passing stool, flatulence, fussing, and Infant Gastrointestinal Symptom Questionnaire (IGSQ) composite scores were not statistically different between any of the formula-feeding groups through to 12 months of age. Infants from Test Group 1 had significantly lower stool consistency scores (indicating softer stools) compared to infants from the Control Group at 2 months ($p<0.05$), but were comparable between formula-fed groups at all other time points. Overall, the odds of adverse events (total, "related", and "related/probably related" to the study product) were comparable between all formula-fed groups at 6 and 12 months of age. When stratified by MedDRA system organ class, "infections and infestations" followed by "gastrointestinal disorders" adverse events were most common for all groups. The odds of a serious adverse event were significantly more likely in Test Group 1 compared to the Control Group at both timepoints ($p<0.05$; most commonly classified as "infections and infestations"), but were not different from the odds in the breastfed reference group and were comparable between Test Group 2 and the Control Group. Furthermore, all serious adverse events in the Test Groups were classified as "unlikely" or "unrelated" to study formulas containing HiMOs.

Taken together, the study investigators concluded that "the co-primary endpoint of growth was met as the mean difference in daily weight gain between baseline and 4 months of age was within the pre-specified non-inferiority margin of -3 g/day when comparing infants fed both Test formulas with those fed the Control formula, and the differences were statistically significantly non-inferior ($p<0.001$)." and "[…] additional growth indices, tolerance outcomes, and safety were similar between all formula-fed groups up to 12 months of age." It is noted that the co-primary safety outcome of growth at 4 months of age is aligned with recommendations from the U.S. Food and Drug Administration as well as the American Academy of Paediatrics for the evaluation of infant formulas with respect to nutritional suitability (AAP, 1988).

Results from benefit-related outcomes of this study are discussed in Part 3.3.3, Section A.2.

Multicentre, Double-Blind, Randomised, Controlled, Parallel-Designed 4-Month Growth, Safety, and Tolerability Study of Formula for Infants Containing 5HMO-Mix (Parschat et al., 2021)

The growth, safety, and tolerability of an infant formula supplemented with a mixture of 5 HiMOs ("5HMO-Mix") during the first 4 months of life has been evaluated in a double-blind, randomised, controlled, multicentre[20] non-inferiority trial (Parschat *et al*., 2021; Clinical Trial Registry NCT03513744). The 4-month intervention period was followed by a 2-month voluntary follow-up period during which parents could choose to continue intervention up to 6 months. Healthy term infants 14 days of age or younger were eligible to participate in the study. Infants whose mother independently and voluntarily chose not to breastfeed were randomised to receive infant formula with or without the addition of the 5HMO-Mix. In parallel, a group of exclusively breastfed infants were enrolled as a reference group.

The basic infant formula providing the macro- and micro-nutrients required for infant nutrition was manufactured in compliance with regulations of the European Union for infant formulae. The Test formula was identical to the basic infant formula apart from the partial replacement of maltodextrin with the 5HMO-Mix containing 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL. Specifically, the 5HMO-Mix was added at a concentration providing 5.75 g/L in the reconstituted infant formula. The concentration of individual HiMOs from the 5HMO-Mix manufactured by Chr. Hansen HMO GmbH (Rheinbreitbach, Germany) added to the Test formula is presented in Table C.2.2-1 below. The reconstituted Control and Test formulas contained similar energy levels (68 and 67 kcal/100 mL, respectively), and identical amounts of protein (1.4 g/100 mL), fat (3.6 g/100 mL), carbohydrates (7.2 g/100 mL), lactose (5.2 g/100 mL), vitamins, and other nutrients.

[20] Subjects were recruited from 12 sites across Germany (2 sites), Italy (5 sites), and Spain (5 sites) from December 2018 to November 2020.

Table C.2.2-1 Concentrations of Individual HMOs from the 5HMO-Mix in the Powdered and Reconstituted Test Infant Formula (Parschat *et al.*, 2021)

HMO	Proportion of 5HMO-Mix (%)	Powdered Test Infant Formula (g/100 g)	Reconstituted Test Infant Formula (g/L)
5HMO-Mix	**100**	**4.35**	**5.75**
2'-FL	52	2.26	2.99
3-FL	13	0.57	0.75
LNT	26	1.13	1.5
3'-SL	4	0.17	0.23
6'-SL	5	0.22	0.28

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactoes; 3'-SL = 3'-sialyllactose; 5HMO-Mix = mixture of 5 HMOs; 6'-SL = 6'-sialyllactose; HMO = human milk oligosaccharide; LNT = lacto-*N*-tetraose.

The primary objective of the trial was to demonstrate that infant formula supplemented with the 5HMO-Mix supports normal non-inferior growth during the first 4 months of age by comparing the mean daily body weight gain after 4 months intervention between the formula-fed groups. Secondary outcomes included other anthropometric measures (absolute data, changes, increments, and WHO growth standard z-scores for weight, length, and head circumference), tolerability (stool frequency and consistency assessed using the Amsterdam Stool Chart), digestive tolerance (regurgitation, vomiting, and flatulence), and behaviour (fussiness, crying, and awakening at night). The primary endpoint was evaluated in the full-analysis dataset (FAS)[21] and the per-protocol dataset (PPS)[22]. Growth parameters were evaluated in the FAS, while all other secondary outcomes were evaluated in the safety dataset (SS)[23].

Overall, 341 infants were enrolled in the study, 225 of which were formula-fed and randomised to the formula groups (113 to the 5HMO-Mix Test Group and 112 to the Control Group); the remaining 116 breastfed infants were allocated to the Reference Group. The study was completed by 265 infants (77.7%), while 76 infants discontinued the study (Control Group: n=21; Test Group: n= 27; Reference Group: n = 28).

The mean daily intake of infant formula on a volume (mL/day) and energy (kcal/day) basis were similar between the Test and Control Groups. The average daily intake of the 5HMO-Mix steadily increased throughout intervention, ranging from 2.6 ± 0.8 g/day at enrolment to 5.2 ± 1.0 g/day at 4 months[24].

The mean daily body weight gain after 4 months of intervention was within the non-inferiority margin of -3 g/day in the Test Group compared to the Control Group for both the FAS and PPS (non-inferiority $p<0.001$). Furthermore, there were no significant differences in any anthropometric measures evaluated between the formula-fed groups throughout intervention.

Stool frequency was similar between the Test Group and breastfed Reference Group from 2 to 4 months; at 4 months, infants from the Control Group passed fewer stools on a daily basis compared to infants from the Test Group ($p=0.0428$) and breastfed infants ($p=0.0136$). A significantly higher frequency of soft stools was observed in the Test Group compared to the Control Group during the first 2 months of intervention ($p<0.05$), while breastfed infants generally had a higher frequency of soft stools compared to both formula-fed groups at most timepoints. There was no difference in flatulence, vomiting, or fussiness without crying between the formula-fed groups. Regurgitation was higher in the Test Group compared to Control from 1 to 4 months ($p<0.05$) but comparable to breastfed infants. Crying was less frequent in the Test Group compared to breastfed infants at most timepoints ($p<0.05$), though no significant difference between the formula-fed groups was observed. Throughout intervention, infants from the formula-fed groups woke less frequently at night compared to breastfed infants ($p<0.05$).

[21] All subjects enrolled in the study who received at least one feeding, had any tolerability data available up to 4 months, and had at least 1 body weight value at baseline and after baseline.

[22] All subjects from the FAS without any major deviations.

[23] All subjects enrolled in the study who received at least one feeding and had any tolerability data available up to 4 months.

[24] Calculated from mean infant formula consumption volumes ranging from 459.7 ± 137.7 mL/day at enrolment to 902.5 ± 170.0 mL/day at 4 months.

The number and intensity of reported adverse events were similar between all 3 groups, and there was no significant difference in adverse events categorised according to the Medical Dictionary for Regulatory Activities (MedDRA) by primary system, organ, and class (SOC) between the formula-fed groups. Among specific adverse events, a higher incidence of genital fungal infection was reported in the Test Group (n=5) compared to Control (n=0; p=0.0290), and haematochezia and plagiocephaly were more frequent in the Test Group compared to the breastfed Reference Group. For haematochezia, the study authors noted that the overall frequency was low (Test Group: n=5; Control Group: n=2; Reference Group: n=2) and could be caused by factors unrelated to the intervention. The majority of serious adverse events were reported in the Control Group (Control Group: n= 9; Test Group: n=3; Reference Group: n = 4). In each of the formula-fed groups, 2 of the reported serious adverse effects were determined to be related to the investigational product. In the Test Group, one subject was hospitalized due to choking and gastroesophageal reflux who later recovered and continued the study, and another subject experienced severe diarrhoea who was treated with hydrolysed milk and removed from the study. Both serious adverse effects reported in the Control Group resulted in the diagnosis of bovine milk protein allergy.

Overall, the study authors concluded that infant formula supplemented with a mixture of 5 HiMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) at concentrations similar to those naturally occurring in human milk supported normal infant growth and was safe and well-tolerated.

Double-Blind, Randomised, Controlled, Growth and Gastrointestinal Tolerance Study of Infant Formula Supplemented with Five HMOs (Lasekan et al., 2022)

The growth and gastrointestinal tolerance of a standard bovine milk-based infant formula with a history of safe use on the U.S. market containing a blend of 5 HMOs was investigated in a double-blind, randomised, controlled, multi-centre trial[25] (Lasekan *et al*., 2022; Clinical Trial Registry NCT04105686). Healthy term infants ≤ 14 days of age at enrolment were randomised to receive the control formula (CF) containing no added HMOs or the experimental formula (EF) supplemented with a blend of 5 HMOs. A non-randomised human milk (HM)-fed group was enrolled in parallel as a reference. Participants consumed the assigned infant formula (or breast milk in HM) as the sole source of nutrition from enrolment to 4 months of age. As the study was conducted during stay-at-home orders from the COVID-19 pandemic, the study protocol was amended to allow alternative anthropometric measurements and extension of the final study visit to up to 183 days of age.

The EF was supplemented with 5.75 g/L of the blend of 5 HMOs composed of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL. The concentration of individual HiMOs in the blend was as follows: 3.0 g/L of 2'-FL, 0.8 g/L of 3-FL, 1.5 g/L of LNT, 0.2 g/L of 3'-SL, and 0.3 g/L of 6'-SL. Both study formulas met the nutrient requirements for infant formula according to U.S. regulations.

The primary outcome of the study was weight gain per day from Days 14 to 119 of life. Secondary outcomes included other anthropometric measures (absolute, interval, and z-scores for comparison to WHO growth standards for weight, length, and head circumference), tolerability (stool frequency, stool consistency, and incidence of spit-up/vomit from feedings), infant behaviour, and adverse events.

Overall, 363 eligible infants were enrolled in the study, 259 of which were formula-fed and randomised to the formula groups (130 receiving EF and 129 receiving CF), and 104 of which were breastfed and allocated to HM Group. The study was completed by 222 infants. A significantly higher proportion of infants from the formula groups exited the study early (EF: n=68.8%; CF: 69.0%) compared to the HM group (88.2%), primarily due to loss at follow-up. Other than a significant greater percentage of infants whose mothers smoked in the EF group than the CF and BM groups, all other baseline characteristics were similar between groups.

There was no significant difference in daily weight gain from enrolment to 4 months of age between the EF and CF groups, nor between the formula-fed groups and the HM group. The EF was determined to be non-inferior to the CF using a margin of 3 g/day. Furthermore, there was no significant difference in absolute and interval weight gain per day between the EF and CF groups for all other timepoints evaluated throughout the study. Similarly, no significant difference in length and head circumference gains was reported from Day 14 to 119 of life between the formula-fed groups. Upon stratification by sex, head circumference gain per day was significantly lower in EF males than CF males at certain study timepoints but remained higher in both formula-

[25] The trial was conducted at 34 study sites throughout the United States.

fed groups compared to the HM group. In both males and females among all study groups, mean weight, length, and head circumference tracked with WHO growth standards.

Study formula intake, number of feedings per day, and percentage of feedings with spit-up or vomit were similar between the formula-fed groups. Compared to infants from the CF group, infants from the EF group had significantly softer stools, greater average number of stools per day, more predominant watery stools, and more predominant yellow stools at multiple timepoints and time intervals evaluated throughout the study, often similar to or approaching that of the HM group. Likewise, average loose stool dimension and constipation dimension scores were significantly higher and lower, respectively, from parent questionnaire responses for infants in the EF group than the CF group.

There was no difference in serious and non-serious adverse events between the three groups. All SAEs reported in infants from the formula-fed groups were determined to be "not related" to the study formulas. By system organ class, the incidence of "General disorder and Administration site administration" was significantly higher in CF than EF, but comparable according to preferred terms (pyrexia and irritability). Significantly less infants from the EF group were seen by healthcare professionals for illness than the CF group from enrolment to Days 56 and 84.

Overall, the study authors concluded that infant formula supplemented the blend of 5 HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) at concentration of 5.75 g/L supported normal growth, tolerance and safety in healthy term infants.

C.3 International Safety Assessments

An overview of safety assessments conducted by other authoritative bodies with comparable regulatory processes on Glycom's that 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation is provided below.

C.3.1 EU

Glycom has submitted applications requesting the use of their 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation as novel foods in the EU pursuant to Regulation (EU) 2015/2283. At the request of the European Commission, the EFSA NDA Panel delivered opinions on 2'-FL/DFL (EFSA, 2019a), LNT (EFSA, 2019b), 6'-SL sodium salt (EFSA, 2020a), and 3'-SL sodium salt (EFSA, 2020b) as novel foods. As the estimated intakes of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL from the proposed conditions in the EU were determined to be unlikely to exceed the intake level of these HMOs from human milk in breastfed infants on a body weight basis, it was concluded across the four Scientific Opinions that 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are safe under their intended uses as a novel food ingredients (EFSA, 2019,a,b; EFSA, 2020a,b). Following these opinions, Commission Implementing Regulations (EU) were issued, authorising the placing on the market of Glycom's 2'-FL/DFL (2019/1979; EU, 2019), LNT (2020/484; EU, 2020), 6'-SL sodium salt (2021/82; EU, 2021a), and 3'-SL sodium salt (2021/96; EU, 2021b) as novel foods under Regulation (EU) 2015/2283 of the European Parliament and of the Council. The EU approvals for 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are specific to Glycom during a 5-year exclusivity period.

Glycom has also submitted an application to extend the use of 2'-FL/DFL and LNT in food supplements for infants pursuant to Regulation (EU) 2015/2283. EFSA recently delivered a scientific opinion on the safety of the extension of use following a request from the European Commission, where the Panel concluded that the use of 2'-FL/DFL and LNT in food supplements for infants is safe under the proposed conditions of use (EFSA, 2022a). EFSA safety assessment of 6'-SL sodium salt and 3'-SL sodium salt already included evaluations of the use of these HiMOs in food supplements for infants (EFSA, 2020a,b).

Furthermore, EFSA has since evaluated the safety of LNT and 3'-SL sodium salt produced by derivative strains of *E. coli* BL21 (DE3) as novel foods (EFSA, 2022b,c).

C.3.2 UK

2'-FL/DFL and LNT are authorised for use as novel foods in the UK under retained EU law following the exit of the UK from the EU (EUR-Lex, 2019, 2020). The conditions of use and specification for 2'-FL/DFL were recently updated in the UK to match those from Implementing Regulation (EU) 2021/50 of 22 January 2021 authorising

an extension of use and a change in the specifications of the novel food '2'-fucosyllactose/difucosyllactose mixture' and amending Implementing Regulation (EU) 2017/2470 (EU, 2021c), approved in the EU after the 31 December 2020 deadline for the preservation of EU legislation into UK domestic legislation. The Food Standards Agency (FSA)/Food Standards Scotland (FSS) reviewed the EFSA opinion and agreed with safety conclusions outlined (FSA/FSS, 2022). The updates in the UK are reflected in The Novel Foods (Authorisations) and Smoke Flavourings (Modification of Authorisations) (England) Regulations 2022 (UK Government, 2022a). Therefore, the conditions of use and specifications for 2'-FL/DFL and LNT are the same in the UK as the EU.

As 6'-SL sodium salt and 3'-SL sodium salt were approved in the EU after the 31 December 2020 deadline, Glycom has submitted applications requesting the use of the sialyllactoses produced by microbial fermentation as novel foods in the UK. The FSA/FSS reviewed the EFSA opinions on 6'-SL sodium salt and 3'-SL sodium salt as novel foods and agreed with safety conclusions outlined (FSA/FSS, 2022). 6'-SL sodium salt and 3'-SL sodium salt were recently authorised for use as novel foods in the UK under The Novel Foods (Authorisations) and Smoke Flavourings (Modification of Authorisations) (England) Regulations 2022 (UK Government, 2022a). The permitted condition of use are laid out in Schedule 3 [Regulation 2(2)(d)], while specifications are laid out in Schedule 7 [Regulation 2(3)(d)], both of which are the same as those authorised in the EU. Schedule 3 was subsequently amended under The Novel Foods (Authorisations) and Smoke Flavourings (Modification of Authorisations) (Amendment) (England) Regulations 2022 to specify that maximum levels are expressed on a 6'-SL and 3'-SL basis (UK Government, 2022b). These regulations amend Commission Implementing Regulation (EU) 2017/2470 establishing the Union list of novel foods in accordance with Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods (EUR-Lex, 2017).

C.3.3 United States

Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation have been determined to be GRAS for their intended conditions of use in the U.S. The GRAS status was notified to the U.S. FDA, and a "no questions" response letter from the U.S. FDA was received by Glycom for 2'-FL/DFL (GRN 815 – U.S. FDA, 2019a), LNT (GRN 833 – U.S. FDA, 2019b), 6'-SL sodium salt (GRN 881 – U.S. FDA, 2020b), and 3'-SL sodium salt (GRN 880 – U.S. FDA, 2020a). As such, Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are not subject to FDA premarket approval and are authorised for use in the U.S. under the conditions of intended use outlined in the GRAS notifications.

C.3.4 Singapore

The SFA has evaluated and authorised the use of Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation in several food categories including infant formula for infants 0 to 6 months of age and infant formula formulas for infants 6 to 12 months of age. SFA authorisation letters are provided in Appendix X (**Commercial Confidential Information**). Authorised conditions of use of 2'-FL/DFL and LNT in infant formula have since been gazetted under Regulation 252 of the Food Regulations (SFA, 2021), while provisions for the use of 6'-SL sodium salt and 3'-SL sodium salt will be incorporated into the next amendment to the Food Regulations.

C.3.5 Israel

The Israel MOH has evaluated and authorised the use of Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation as novel foods in several food categories, including infant formula and follow-formula (Israel MOH, 2022a,b,c,d).

C.3.6 Brazil

ANVISA has evaluated and authorised the use of Glycom's LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation in several food categories including infant formula for infants 0 to 6 months of age and follow-up formula for infants and young children 6 to 36 months of age. ANVISA authorisation letters are provided in Appendix X (**Commercial Confidential Information**).

D. Information on Dietary Intake of the Nutritive Substance

D.1 List of Food Groups or Foods Likely to Contain the Nutritive Substance

2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are intended to be added alone or in combinations to infant formula products (as defined in Standard 2.9.1).

D.2 Proposed Maximum Levels in Food Groups or Foods

Maximum proposed use levels of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt for addition to infant formula products are presented in Table D.2-1. Maximum use levels are expressed on a HiMO basis rather than on an ingredient basis. Notably, 2'-FL/DFL is intended to be used as an alternative source of 2'-FL, serving as a direct substitute for 2'-FL already approved (alone or in combination with LNnT – Schedule 29: S29—5).

Table D.2-1 Maximum Proposed Use Levels of 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3'-SL Sodium Salt (on a Specified HiMO Basis) in Infant Formula Products

Ingredient	Specified HiMO(s)	Maximum Amount of specified HiMO(s)	
		per 100 KJ	per L
2'-FL/DFL Mixture	2'-FL + DFL	96 mg	2.4
LNT	LNT	32 mg	0.8
6'-SL sodium salt	6'-SL	16 mg	0.4
3'-SL sodium salt	3'-SL	8 mg	0.2

2'-FL/DFL = 2'-fucosyllactose/difucosyllactose mixture; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; LNT = lacto-*N*-tetraose.

The maximum use levels of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are proposed on the basis of providing similar levels of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL as those occurring on average in human milk (see Part 3.6.2, Section A.3.1.2, of the application). The intention is to achieve dietary intake levels of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL from infant formula products that are within natural intake levels of these HMOs from human milk.

D.3 Likely Level of Consumption

Dietary intake estimates calculated herein are considered applicable to the use of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt individually or in combinations in infant formula products as:

- 2'-FL, DFL, LNT, 6'-SL, and 3'-SL naturally occur together in human breast milk; and
- Maximum proposed use levels of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt in infant formula products, expressed on a HiMO basis, are based on mean concentrations of corresponding HMOs naturally occuring in human milk.

D.3.1 Australia/New Zealand

FSANZ previously estimated the dietary intakes of 2'-FL and LNnT from infant formula and follow-on formula in infants 3 and 9 months of age in their risk assessment report for Application A1155 (FSANZ, 2019c). The maximum use level of 2'-FL that was evaluated (96 mg/100 KJ or 2.4 g/L) is the mean concentration of 2'-FL in mature Secretor milk (60+ days post-partum), currently authorized for 2'-FL in Schedule 29. Estimates were based on infant model diets, where consumption was derived based on energy contents of infant formula and follow-on formula. High-level dietary intakes at the 90th percentile were estimated by doubling the mean exposure as only mean food consumption amounts were available from model diets. 2'-FL intake estimates and default values are reproduced in Table D.3.1-1.

For infants consuming infant formula products for special dietary use, infants with similar energy requirements and consuming products with similar energy content to those from the model diets are expected to have

similar 2'-FL intakes, while infants with similar energy requirements but consuming products with a higher energy content are expected to have lower 2'-FL intakes.

As the maximum proposed use level of 2'-FL/DFL is the same as the maximum authorised use level of 2'-FL alone or in combination with LNnT in Schedule 29 of the Code (*i.e.,* 96 mg/100 KJ or 2.4 g/L), dietary intakes of 2'-FL previously calculated by FSANZ (2019c) remain relevant to the current application and confirm the safe intake of 2'-FL from the 2'-FL/DFL mixture.

Table D.3.1-1 Estimated Dietary Intakes of 2'-FL for Infants Aged 3 Months and 9 Months from Infant Formula / Follow on Formula (Reproduced from FSANZ, 2019c)

	Units	3 Months[c]	9 Months[c]
Recommended energy intake[a]	kJ/kg bw/day	343	330
P50 Body Weight[b]	kg	6.4	8.9
Recommended energy intake	kJ/day	2,195	2,937
100 % of energy requirements[c]	kJ/day	2,195	n/a
50 % of energy requirements[c]	kJ/day	n/a	1,469
Mean dietary intake of 2'-FL$_{micro}$	g/day	2.1	1.4
	g/kg bw/day	0.33	0.16
P90 dietary intake of 2'-FL$_{micro}$	g/day	4.2	2.8
	g/kg bw/day	0.66	0.32

2'-FL = 2'-fucosyllactose; bw = body weight; P90 = 90th percentile.
[a] (FAO/WHO/UNU, 2004)
[b] (WHO, 2006)
[c] Energy content of infant formula and follow on formula is 264 kJ/100 g formula (FSANZ, 2016), with the proposed concentration of 2'-FL $_{micro}$ in infant formula and follow-on formula being 96 mg/100 kJ.

Mean dietary intakes of DFL, LNT, 6'-SL, and 3'-SL were estimated using the same default values for infant energy requirements, and by applying maximum proposed use levels of the specified HiMOs in infant formula products (see Table D.2-1 of the previous Section). High-level dietary intakes at the 90th percentile were estimated by doubling the mean exposure. As 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are proposed for use in infant formula products, calculations are provided for infants 3 and 9 months of age only[26]. The resulting mean and 90th percentile estimated daily intakes of DFL, LNT, 6'-SL, and 3'-SL from infant formula products in infants 3 and 9 months of age are presented in Table D.3.1-2.

Similar to FSANZ's previous conclusions on 2'-FL for infants consuming infant formula products for special dietary use, infants with similar energy requirements and consuming products with similar energy content to those from the model diets are expected to have similar DFL, LNT, 6'-SL and 3'-SL intakes, while infants with similar energy requirements but consuming products with a higher energy content are expected to have lower intakes of these HiMOs.

[26] In the risk assessment report for A1155, calculations for infants 12 months of age were specific to formulated supplementary foods for young children (FSFYC), which are not within the scope of the current application.

Table D.3.1-2 Calculation of Estimated Dietary Intakes of DFL, LNT, 6'-SL, and 3'-SL from Infant Formula / Follow on Formula for Infants Aged 3 Months and 9 Months[a]

Ingredient	Maximum Proposed Use Level of Specified HiMO(s) in Infant Formula Products	Statistic	Units	Dietary Intake from Secretor Human Milk	
				3 Months	9 Months
DFL	96 mg/100 KJ of 2'-FL + DFL, assuming 12% DFL[b]	Mean	g/day	0.25	0.17
			g/kg bw/day	0.04	0.02
		P90[c]	g/day	0.50	0.34
			g/kg bw/day	0.08	0.04
LNT	32 mg/100 KJ of LNT	Mean	g/day	0.70	0.47
			g/kg bw/day	0.11	0.05
		P90[c]	g/day	1.4	0.94
			g/kg bw/day	0.22	0.11
6'-SL sodium salt	16 mg/100 KJ of 6'-SL	Mean	g/day	0.35	0.24
			g/kg bw/day	0.05	0.03
		P90[c]	g/day	0.70	0.47
			g/kg bw/day	0.11	0.05
3'-SL sodium salt	8 mg/100 KJ of 3'-SL	Mean	g/day	0.18	0.12
			g/kg bw/day	0.03	0.01
		P90[c]	g/day	0.35	0.24
			g/kg bw/day	0.05	0.03

2'-FL/DFL = 2'-fucosyllactose and difucosyllactose mixture; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; bw = body weight; DFL = difucosyllactose; HiMO = human-identical milk oligosaccharide; LNT = lacto-*N*-tetraose; P90 = 90th percentile.

[a] Calculated based on default values for infant energy requirements previously used by FSANZ (2019c).

[b] Highest DFL content on an ingredient basis, and mean DFL content on a HiMO basis, from 5 independent representative batches of 2'-FL/DFL (see Appendix V).

[c] Dietary intakes at the 90th percentile were calculated by doubling the mean intake value.

It is herein noted that the estimated intake levels were derived using the maximum proposed use level of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt on a HiMO basis while the actual inclusion levels may be less when it is formulated into finished products. As such, even with these conservative assumptions, the intake of DFL, LNT, 6'-SL, and 3'-SL remain within ranges that have been reported from its consumption as an inherent constituent of human milk (see Part 3.6.2, Section A.3.1.3, of this application).

D.3.2 European Union

EFSA has provided its own estimates of the daily intake of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL in infants up to 16 weeks of age based on maximum use levels that were authorised in the EU (EFSA, 2019a,b; EFSA, 2020a,b). As described in Section D.5, authorised use levels of LNT, 6'-SL sodium salt, and 3'-SL sodium salt in infant formula in the EU are the same as those proposed for infant formula products in Australia and New Zealand in the current application, but slightly lower in the EU for 2'-FL/DFL. Estimates generated by EFSA were based on an infant formula high level consumption value of 260 mL/kg body weight/day during the first weeks of life (EFSA, 2017). These estimates are reproduced in Table D.3.2-1 below.

Table D.3.2-1 Summary of EFSA Daily Intakes Estimates for 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3'-SL Sodium Salt in Infants up to 16 Weeks of Age

Novel Food	EU Use Level (g/L)	Mean HiMO Content	High Intake (mg/kg bw/day)[a]		Reference
			Novel Food Basis	HiMO Basis	
2'-FL/DFL	1.6	81% 2'-FL; 11% DFL	416	337 for 2'-FL; 46 for DFL	EFSA (2019a)
LNT	0.8	78%	208	162	EFSA (2019b)
6'-SL sodium salt	0.4	-	104	-	EFSA (2020a)
3'-SL sodium salt	0.2	-	52	-	EFSA (2020b)

2'-FL = 2'-fucosyllactose; 2'-FL/DFL = 2'-fucosyllactose and difucosyllactose mixture; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; bw = body weight; DFL = difucosyllactose; EFSA = European Food Safety Authority; EU = European Union; HiMO = human-identical milk oligosaccharide; LNT = lacto-*N*-tetraose.

[a] Based on the EFSA high consumption level value of 260 mL/kg bw/day for infant formula.

It is noted that the anticipated daily intakes of LNT, 6'-SL sodium salt, 3'-SL sodium salt (expressed on a novel food basis) for infants up to 16 weeks of age calculated by EFSA on an infant formula consumption level basis (208, 104, and 57 mg/kg body weight/day, respectively – see Table D.3.2-1) are similar to the 90th percentile daily intake estimates of LNT, 6'-SL, 3'-SL for infants 3 months of age calculated using the FSANZ approach on an energy requirement basis (~220, 110, 50 mg/kg body weight/day, respectively – see Table D.3.1-2).

As previously mentioned, the proposed use level of 2'-FL/DFL in Australia and New Zealand (2.4 g/L) is slightly higher than the authorised use level of 2'-FL/DFL in the EU (1.6 g/L), to allow direct substitution of the already approved 2'-FL in Australia and New Zealand for 2'-FL/DFL. As a result, the anticipated daily intake of DFL for infants 3 months of age calculated using the FSANZ approach (~80 mg/kg body weight/day – see Table D.3.1-2) is proportionally higher than that calculated by EFSA for DFL on a HiMO basis (46 mg/kg body weight/day – see Table D.3.2-1).

D.4 Percentage of Food Group to Use Nutritive Substance

In deriving the estimated intake of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt from their proposed uses in infant formula products, it can be assumed, as the most conservative measure, that the specified HiMOs will be added to all infant formula products marketed in Australia/New Zealand (including both powdered and ready-to-feed formulations).

Although it is unlikely that the inclusion of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt will have 100% market penetration, in reality, the application of this assumption in the exposure estimate will provide a conservative estimate of the intake of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL under the proposed conditions of use.

It is further noted that Glycom is seeking exclusive permission to market their 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt ingredients as foods produced using gene technology (see Part 3.1.1, Section I, of this application).

D.5 Use in Other Countries

Maximum authorised levels of use of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt for addition to infant formula products in countries with comparable regulatory processes to Australia and New Zealand (*i.e.*, the EU, the U.S., Singapore, Israel, and Brazil) are summarised in Table D.5-1. In addition to infant formula products, 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are also approved for use in baby foods and conventional foods in these jurisdictions, as well as in foods for specifical medical purposes and food supplements for young children and the general population in the EU and associated countries that follow EU law.

Maximum proposed levels of Glycom's LNT (0.8 g/L on a LNT basis), 6'-SL sodium salt (0.4 g/L on a 6'-SL basis), and 3'-SL sodium salt (0.2 g/L on a 3'-SL basis) for use in infant formula products in Australia and New Zealand are the same as the highest authorised use levels for these HiMOs in infant formula in countries with

comparable regulatory processes (Table D.5-1). As 2'-FL/DFL is intended to be used as an alternative to other 2'-FL sources already authorised for use in infant formula products in Australia and New Zealand (*i.e.,* 2'-FL alone or in combination with LNnT at up to 2.4 g/L), the maximum proposed level of 2'-FL/DFL is higher than the highest authorised level of this HiMO in other countries (Table D.5-1).

Table D.5-1 Summary of Authorised Maximum Use Levels for 2'-FL/DFL, LNT, 6'-SL Sodium Salt, and 3' SL Sodium Salt in Formula Products in Other Countries

Substance	EU, Switzerland, UK	U.S.	Singapore	Israel	Brazil
2'-FL/DFL	Infant formula (≤6 m) 1.6 g/L Follow-on formula (6-12 m) 1.2 g/L	Infant formula (≤12 m) 1.6 g/L Toddler formula (>12 m) 1.2 g/L	Infant formula (≤6 m) 1.6 g/L Infant formula (6-12 m) 1.2 g/L	Infant formula (≤6 m) 1.6 g/L Follow-on formula (6-12 m) 1.2 g/L	-
LNT	Infant formula (≤6 m) 0.8 g/L Follow-on formula (6-12 m) 0.6 g/L	Infant formula (≤12 m) 0.8 g/L Toddler formula (>12 m) 0.6 g/L	Infant formula (≤6 m) 0.8 g/L Infant formula (6-12 m) 0.6 g/L	Infant formula (≤6 m) 0.8 g/L Follow-on formula (6-12 m) 0.6 g/L	Infant formula (≤6 m) 0.8 g/L Follow-up formula (>6 m) 0.6 g/L
6'-SL sodium salt	Infant formula (≤6 m) 0.4 g/L Follow-on formula (6-12 m) 0.3 g/L	Infant formula (≤12 m) 0.4 g/L Toddler formula (>12 m) 0.3 g/L	Infant formula (≤6 m) 0.4 g/L Infant formula (6-12 m) 0.3 g/L	Infant formula (≤6 m) 0.4 g/L Follow-on formula (6-12 m) 0.3 g/L	Infant formula (≤6 m) 0.4 g/L Follow-up formula (>6 m) 0.3 g/L
3'-SL sodium salt	Infant formula (≤6 m) 0.2 g/L Follow-on formula (6-12 m) 0.15 g/L	Infant formula (≤12 m) 0.2 g/L Toddler formula (>12 m) 0.15 g/L	Infant formula (≤6 m) 0.2 g/L Infant formula (6-12 m) 0.15 g/L	Infant formula (≤6 m) 0.2 g/L Follow-on formula (6-12 m) 0.15 g/L	Infant formula (≤6 m) 0.2 g/L Follow-up formula (>6 m) 0.15 g/L

6'-SL = 6'-sialyllactose; 3'-SL = 3'-sialyllactose; EU = European Union; U.S. = United States.

D.6 Where Consumption has Changed, Information on Likely Consumption

Not applicable.

E. Nutritional Impact of a Vitamin or Mineral

Not applicable.

F. Nutritional Purpose of the Use of the Substance in Each Food

The addition of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt to infant formula products is at a level similar to (and no higher than) the levels occurring in human milk. The inclusion of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt is to solely bring available infant formula products closer in composition to human 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).

Infant formula products that contain 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt would be required to meet the compositional requirements that have been already established in Standard 2.9.1 and thus would be nutritionally balanced. No anti-nutritional effects (*i.e.*, reductions in the availability of nutrients) are expected following the consumption of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt.

G. Potential Impact on Consumer Understanding and Behaviour

G.1 Consumer Awareness and Understanding

According to the most recent Australian National Breastfeeding Strategy, women are aware of the benefits of breastfeeding but face societal and cultural barriers to breastfeeding (COAG Health Council, 2019). Australian and New Zealand health advisories and breastfeeding associations have conducted campaigns to raise public awareness of the benefits of breastfeeding. For example, the House of Representatives Standing Committee on Health and Ageing considered the health benefits of breastfeeding and recommended the funding of a national education campaign to highlight the health benefits of breastfeeding to mothers and babies (Commonwealth of Australia, 2007). The Australian National Breastfeeding Strategy 2010–2015 was developed in response to these recommendations (among others). As a result, the National Health and Medical Research Council (NHMRC) released the *Infant Feeding Guidelines: Information for Health Workers* providing updated advice for both health professionals and parents on breastfeeding and infant feeding, including a summary of the evidence on the health benefits of breastfeeding (Commonwealth of Australia, 2016). The 2019 Australian National Breastfeeding Strategy includes additional information on the benefits of breastfeeding, including the citation of a review on the impact of infant feeding on the development of the infant microbiome (Mueller *et al*. 2015). Therefore, it is expected that the majority of health professionals and parents/caregivers in Australia and New Zealand are aware of the positive health benefits from breastfeeding.

Historically, inulin-type fructans (previously inulin-derived substances or IDS), alone or in combination with galacto-oligosaccharides, have been permitted for addition to infant formula products, infant foods and formulated supplementary foods for young children (FSFYC) in Australia and New Zealand (FSANZ, 2008 – P306; FSANZ, 2013b – A1055). The purpose of the addition of these oligosaccharide preparations to infant and follow-on formula is to mimic the effects of oligosaccharides that occur naturally in breast milk (FSANZ, 2008), and to better align the stool characteristics of formula-fed infants with the softer stools typically associated with breastfed infants (FSANZ, 2013). The manufactured human milk oligosaccharides 2'-FL and LNnT, demonstrated to be chemically and structurally identical to their counterparts in human milk, were first approved for addition to infant formula products by FSANZ in December 2019 (FSANZ, 2019a – A1155). Several other manufactured 2'-FL from new GM sources have since been approved by FSANZ (FSANZ, 2021b – A1190; FSANZ 2022a – A1233), while an application to permit the use of manufactured 2'-FL in combination with galacto-oligosaccharides and/or inulin-type fructans in infant formula products is currently under evaluation by FSANZ (FSANZ, 2022b – A1251). According to a search of the Mintel Global New Products Database (GNPD), six infant formula products containing 2'-FL alone or in combination with LNnT on the Australia and/or New Zealand markets were identified, summarised in Table G.1-1 below (results available upon request). As the commercial use of manufactured HMOs in infant formula products is relatively new in Australia and New Zealand, it is not expected that the majority of Australian and New Zealand consumers will be aware of HMOs and understand their benefits. As health professionals become more familiar with these ingredients over time and their role in the nutrition of formula-fed infants, it is anticipated that such products may be recommended to mothers/caregivers who are unable to breastfeed or who choose not to breastfeed.

No data on consumer awareness and understanding of HMOs were identified from peer-reviewed scientific journals. Several market analysis reports suggest increased market growth as a result of increased awareness of the health benefits of HMOs[27]. Glycom will continue to monitor data and evidence related to consumer awareness and understanding of HMOs throughout FSANZ's evaluation of this application.

[27] Human Milk Oligosaccharides Market Size | Industry Report, 2027 (grandviewresearch.com); Human Milk Oligosaccharides (HMO) Market Size, Share & Forecast, 2028 (alliedmarketresearch.com)

Table G.1-1 Commercialised Infant Formula and Follow-On Formula Products Containing 2'-FL with or without LNnT in Australia and New Zealand

Product	Description
	**Alula Advance+ Stage 1 Newborn Premium Infant Formula**, containing 100 mg of 2'-FL and 50 mg of LNnT per 100 mL.
	**Alula Advance+ Stage 2 Follow-On Premium Infant Formula**, containing 100 mg of 2'-FL and 50 mg of LNnT per 100 mL.
	**Future Gradulac Gentle Stage 1 Newborn Infant Nourish PP+ Premium Formula**, containing 82 mg of 2'-FL per 100 mL.
	Future Gradulac Gentle Stage 2 Follow-On Nourish PP+ Premium Formula, containing 82 mg of 2'-FL per 100 mL.
	**Oli6 Stage 1 Goat Milk Infant Formula**, containing 24 mg of 2'-FL and 6 mg of LNnT per 100 mL.
	**Oli6 Stage 2 Goat Follow on Formula**, containing 24 mg of 2'-FL and 6 mg of LNnT per 100 mL.

Table G.1-1 Commercialised Infant Formula and Follow-On Formula Products Containing 2'-FL with or without LNnT in Australia and New Zealand

Product	Description
	**Biostime SN-2 Bio Plus Stage 1 HPO Infant Formula**, containing 0.94 g of 2'-FL per 100 g.

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

G.2 Actual or Potential Behaviour of Consumers

Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt have recently been commercialised into infant formula products in over 20 countries[28]. The anticipated behaviour of Australian and New Zealand consumers in response to the market entry of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt is expected to be comparable to that already seen globally for non-digestible oligosaccharides.

Australia's National Health and Medical Research Council (NHMRC) and New Zealand's Ministry of Health recommend exclusive breastfeeding for around the first 6 months and then for breastfeeding to continue alongside complementary foods for 1 year, or as long as mother and child desire. Infant formula is the only suitable and safe alternative to breastfeeding to meet an infant's primary nutritional needs. As the purpose of adding 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt to infant formula products is to make its composition closer to that of human milk, which is considered the gold-standard of infant feeding, it is expected that such products will be well-received by mothers/caregivers who are unable to breastfeed or who choose not to breastfeed.

G.3 Demonstration of no Adverse Effects on Any Population Groups

2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt produced by microbial fermentation are structurally identical to their naturally occurring counterparts that are present in human milk. The proposed use levels in infant formula products are based on levels observed in human milk; therefore, the dietary intake of 2'-FL/DFL, LNT, 6'-SL sodium salt (on a 6'-SL basis), and 3'-SL sodium salt (on a 3'-SL basis) from formula would result in a comparable exposure to that of breastfeeding infants, and accordingly are considered safe based on their history of consumption.

A comprehensive discussion on the toxicological and clinical studies conducted on 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt, and safety of the production strains, is provided in Section C. No data received to current day indicate the target population group in Australia and New Zealand would be adversely affected. Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt have already been commercialised into infant formula products in several markets (as indicated in Section F.2), from which post-market surveillance data have not indicated any untoward effects attributed to the intake of these HiMOs.

Furthermore, the 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt ingredients and their 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. 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are produced from lactose as a starting material, which is derived from milk (listed in S9—3). Infant formula products containing the HiMOs will be labelled in accordance to mandatory declarations requirements set out in Division 3 of Standard 1.2.3 of the Code.

No significant safety concern has been identified from post-market surveillance data collected over a period of 5 years (from 2017 to 2021) from 36 countries in which stage 1 infant formulae containing 2'-FL and LNnT have

[28] Austria, Chile, Colombia, Czech Republic, Denmark, Finland, France, Germany, Greece, Hong Kong, Hungary, Italy, Mexico, Portugal, Romania, Spain, Sweden, Slovakia, Ukraine, and Vietnam (see Appendix XI).

been commercialised. Similarly, no significant safety concern has been identified from post-market surveillance data collected over the past year (2021) of newly commercialised infant formula products containing HiMO mixtures of 2'-FL/DFL, LNT, 6'-SL, and 3'-SL in 21 countries. These data, provided in Appendix XI **(Confidential Commercial Information)**, demonstrate a safe history of use in infant formula of HiMOs authorised and proposed for use in infant formula products in Australia and New Zealand.

PART 3.5.1 – FOODS PRODUCED USING GENE TECHNOLOGY

A. Technical Information on the Food Produced using Gene Technology

A.1 Nature and Identity

The four HiMO product that are the subject of this application (2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt) meet the definition of "Food produced using gene technology", as defined in Standard 1.5.2, on the basis that it is considered a "food which has been derived or developed from an organism which has been modified by gene technology" (FSANZ, 2018).

Specifically, 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are produced from derivatives of *E. coli* strain K-12, optimised for general oligosaccharide expression and modified with the introduction of well-defined, synthesised DNA sequences, to enable the metabolism of complex carbohydrates and the conversion of starting carbohydrates into oligosaccharides that are identical to those in human milk. Glycom's platform strain for the manufacture of a number of HiMOs, including 2'-FL and LNnT already authorised in Australia and New Zealand, has been modified for the production of each of the four HiMO products through the introduction of genes necessary for the biosynthesis of 2'-FL and DFL, LNT, 6'-SL, and 3'-SL. The production strains are highly stable and reliable and provide high titres of the HiMOs. All four production strains have been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Germany. Deposition certificates are provided in Appendix XII **(Confidential Commercial Information)**.

The proposed definition of the source organism for the manufacture of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt is compared in Table A.1-1 to source organism definitions for Glycom's 2'-FL and LNnT authorisations laid down in Schedule 26. Briefly:

- The 2'-FL/DFL source organism meets the currently approved definition of the source organism for Glycom's 2'-FL [S26—3 (7) – associated with Application A1155].
- The LNT source organism is similar to the currently approved definition of the source organism for LNnT [S26—3 (7)], the only difference being the glycosylation specificity of the galactosyltransferase enzyme (β1-3 *versus* β1-4 linkage, respectively).
- The 6'-SL and 3'-SL source organisms have biosynthetic genes for sialylation. Biosynthetic genes for 6'-SL and 3'-SL are similar, the only difference being the glycosylation specificity of the sialyltransferase enzyme (α2-6 *versus* α2-3 linkage, respectively).

Table A.1-1 Comparison of Authorised and Proposed Source Organism Definitions for Food Produced Using Gene Technology of Microbial Origin in Schedule 26

	Substance	Source
Authorised		
1	**2'-Fucosyllactose**	*Escherichia coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori* (associated with Application A1155)
2	**Lacto-*N*-neotetraose**	*Escherichia coli* K-12 containing the gene for beta-1,3-*N*-acetylglucosaminyltransferase from *Neisseria meningitides* and the gene for beta-1,4-galactosyltransferase from *Helicobacter pylori*
Proposed		
X	**2'-Fucosyllactose/ difucosyllactose mixture**	*Escherichia coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori*

Table A.1-1 Comparison of Authorised and Proposed Source Organism Definitions for Food Produced Using Gene Technology of Microbial Origin in Schedule 26

Substance	Source
✗ **Lacto-*N*-tetraose**	*Escherichia coli* K-12 containing the gene for beta-1,3-*N*-acetylglucosaminyltransferase from *Neisseria meningitides* and the gene for beta-1,3-galactosyltransferase from *Helicobacter pylori*
✗ **6'-Sialyllactose sodium salt**	*Escherichia coli K-12* containing the gene for alpha-2,6-sialyltransferase from *Photobacterium damsela* and CMP-Neu5Ac synthetase, Neu5Ac synthase, *N*-acetylglucosamine-6-phosphatase epimerase from *Campylobacter jejuni*
✗ **3'-Sialyllactose sodium salt**	*Escherichia coli K-12* containing the gene for alpha-2,3-sialyltransferase from *Neisseria meningitides* and CMP-Neu5Ac synthetase, Neu5Ac synthase, *N*-acetylglucosamine-6-phosphatase epimerase from *Campylobacter jejuni*

A.2 History of Use of Host and Donor Organisms

The 2'-FL/DFL, LNT, 6'-SL, and 3'-SL production strains are derived from the same platform strain that has served as the host for the engineering all of Glycom's HiMO production strains, including 2'-FL and LNnT already authorised for use in Australia and New Zealand under Schedule 26 as food produced using gene technology of microbial origin (FSANZ, 2021a).

During manufacture, 2'-FL/DFL, LNT, 6'-SL, and 3'-SL are secreted extracellularly by the production strain, following which intact cells are entirely removed *via* filtration and the HiMOs are isolated from the fermentation medium through a series of purification steps (as described in Part 3.3.3, Section B.4, of this application).

The specific details on the donor organisms from which the genetic elements are derived and characteristics of the host organism are provided below.

A.2.1 Host Organism

The host organism (*E. coli* K-12) is the same as that used to make Glycom's 2'-FL and LNnT as specified currently in Schedule 26. Briefly, *E. coli* K-12 was specifically developed and recognised as a "safety strain" for molecular biological research in the 1970s (Manning *et al.*, 1977; Smith, 1978), and was among the first organisms in history of modern sequencing technologies for which the whole genome sequence became available (Blattner *et al.*, 1997). *E. coli* K-12-derived strains cannot colonise in the human gastrointestinal system, and do not produce protein-type toxins (U.S. EPA, 1997). *E. coli* K-12 derivatives are today among the preferred microorganisms for industrial biotechnology with wide application scope (Chen *et al.*, 2013; Theisen and Liao, 2017).

A.2.2 Donor Organism

As indicated above, Glycom's HiMO platform strain has been modified for the production of 2'-FL/DFL, LNT, 6'-SL, and 3'-SL through the introduction of genes necessary for their biosynthesis.

Specific modifications applied to the 2'-FL/DFL, LNT, 6'-SL, and 3'-SL production strains, including biochemical pathway by which the production strains generate the HiMOs from the starting substrate (D-lactose) and carbon source (D-glucose or alternatively glycerol or sucrose), are described in more detail below.

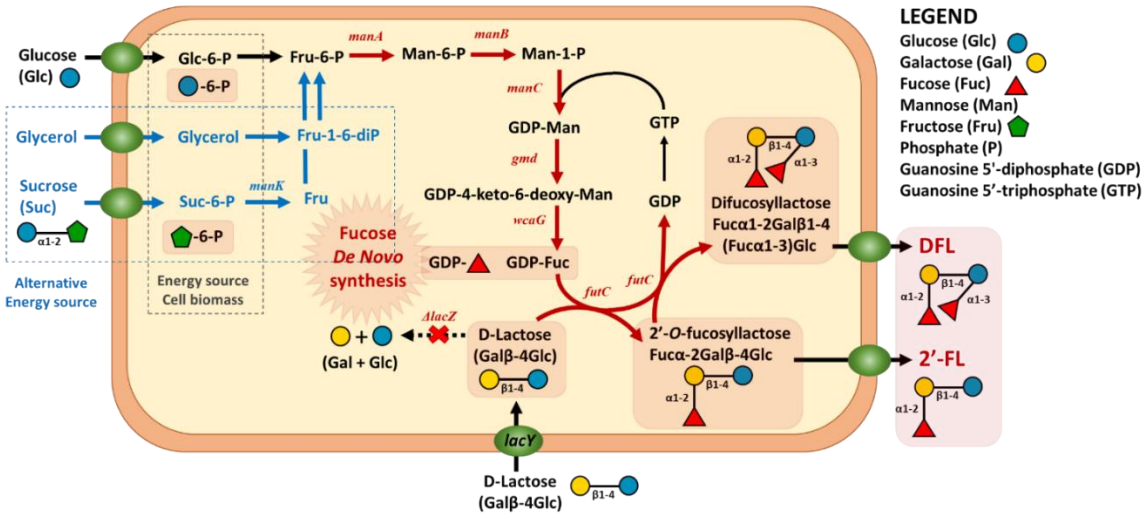
Notably, donor genes were not isolated or directly amplified from the donor organisms but rather derived from *de novo* DNA synthesis based on defined DNA sequences obtained from bioinformatic databases. Furthermore, the identity and function of all enzymes involved in the biosynthesis of 2'-FL/DFL, LNT, 6'-SL, and 3'-SL are well-characterised. Therefore, the introduction of these genes would not confer toxicogenic/pathogenic properties to the host organism.

2'-FL/DFL

The 2'-FL/DFL mixture is manufactured using the same source microorganism cited for the manufacture of 2'-FL on its own in Schedule 26 previously evaluated by FSANZ (Application A1155). Namely, the source organism used to produce 2'-FL as well as the 2'-FL/DFL mixture is *E. coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori*. Alpha-1,2-fucosyltransferase is the enzyme that catalyses the fucosylation of D-lactose (the substrate) to form 2'-FL, and the subsequent fucosylation of 2'-FL forms DFL. Variations in the processing conditions determine whether the 2'-FL/DFL mixture or 2'-FL on its own (with low DFL) is produced; these include deliberate control of the concentrations of the starting substrate (*i.e.*, D-lactose), and modifications of downstream purification processes (*i.e.*, inclusion of a crystallisation step to remove DFL).

Figure A.2.2-1 shows the biochemical pathway by which the 2'-FL/DFL production strain generates 2'-FL (and in turn DFL from 2'-FL) using D-lactose as the starting substrate and D-glucose (or optionally D-sucrose or glycerol) as a carbon source.

Figure A.2.2-1 Engineered Pathway for 2'-FL and DFL Biosynthesis in the *Escherichia coli* K-12 Derived Production Strain

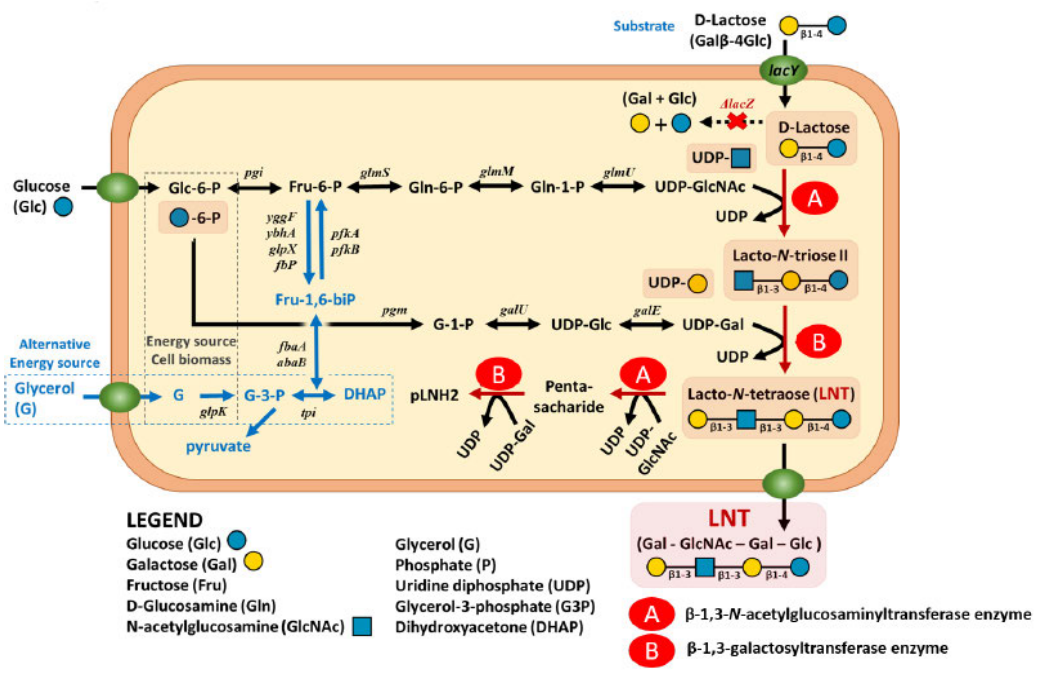


LNT

Modifications applied to the LNT production strain are similar to those cited for the manufacture of LNnT in Schedule 26 previously evaluated by FSANZ (Application A1155). The first gene, derived from *Neisseria meningitides*, expresses beta-1,3-N-acetylglucosaminyltransferase, an enzyme that catalyses the conversion of D-lactose to lacto-*N*-triose II. The second gene, derived from *Helicobacter pylori*, expresses beta-1,3-galactosyltransferase, an enzyme that catalyses the conversion of lacto-*N*-triose II to LNT. Therefore, both source organisms for LNT are the same as those cited in Schedule 26 for the manufacture of LNnT, the only difference being the gene obtained from *Helicobacter pylori*, expressing either beta-1,4-galactosyltransferase (LNnT), or beta-1,3-galactosyltransferase (LNT).

Figure A.2.2-2 shows the biochemical pathway by which the LNT production strain generates LNT using D-lactose as the starting substrate and D-glucose (or optionally D-sucrose or glycerol) as the carbon source.

Figure A.2.2-2 Engineered Pathway for LNT Biosynthesis in the *E. coli* K-12 Derived Production Strain

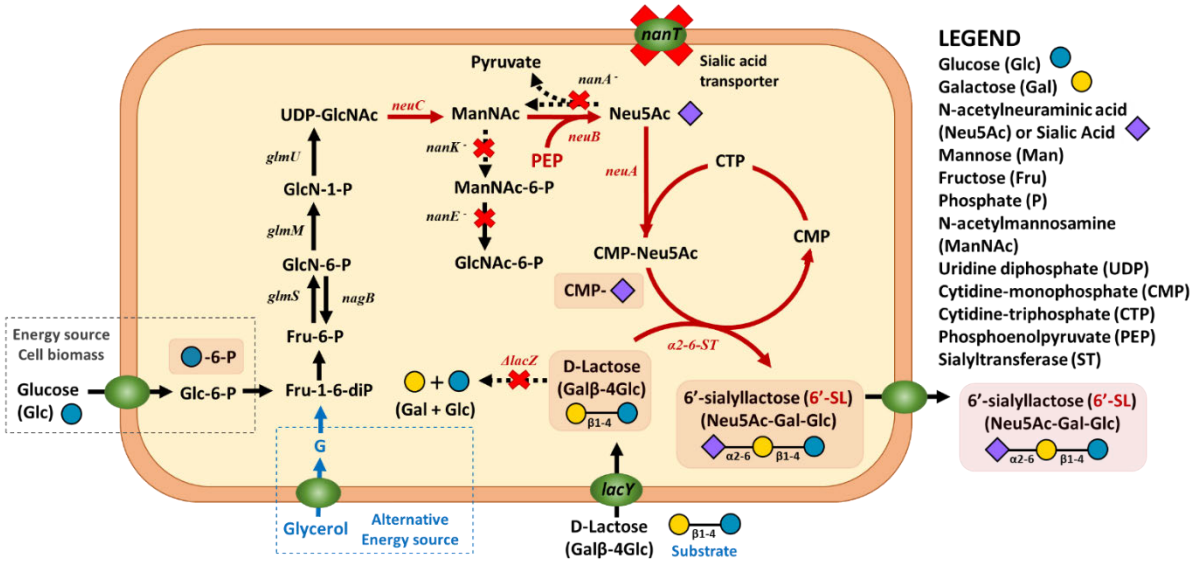


6'-SL

The first modification of the 6'-SL production strain is the overexpression of a gene cluster derived from *Campylobacter jejuni* expresses 3 enzymes involved in *de novo* synthesis of cytidine monophosphate (CMP)-sialic acid (Neu5Ac): a Neu5Ac synthase, a *N*-acetylglucosamine-6-phosphate-epimerase, and a CMP-Neu5Ac synthetase. The second modification is the overexpression of a gene derived from *Photobacterium damsela* that expresses α-2,6-sialyltransferase, an enzyme that catalyses the transfers a Neu5Ac unit from the intracellular CMP-Neu5Ac pool to the 6'-position of the D-galactose residue of D-lactose (starting substrate) in a α stereospecific manner to form the trisaccharide 6'-SL (α2,6-sialyllactose), with concurrent release of CMP.

Figure A.2.2-3 shows the biochemical pathway by which the 6'-SL production strain generates 6'-SL using D-lactose as the starting substrate and D-glucose (or alternatively D-sucrose or glycerol) as the carbon source.

Figure A.2.2-3 Engineered Pathway for 6'-SL Biosynthesis in the *E. coli* K-12 Derived Production Strain

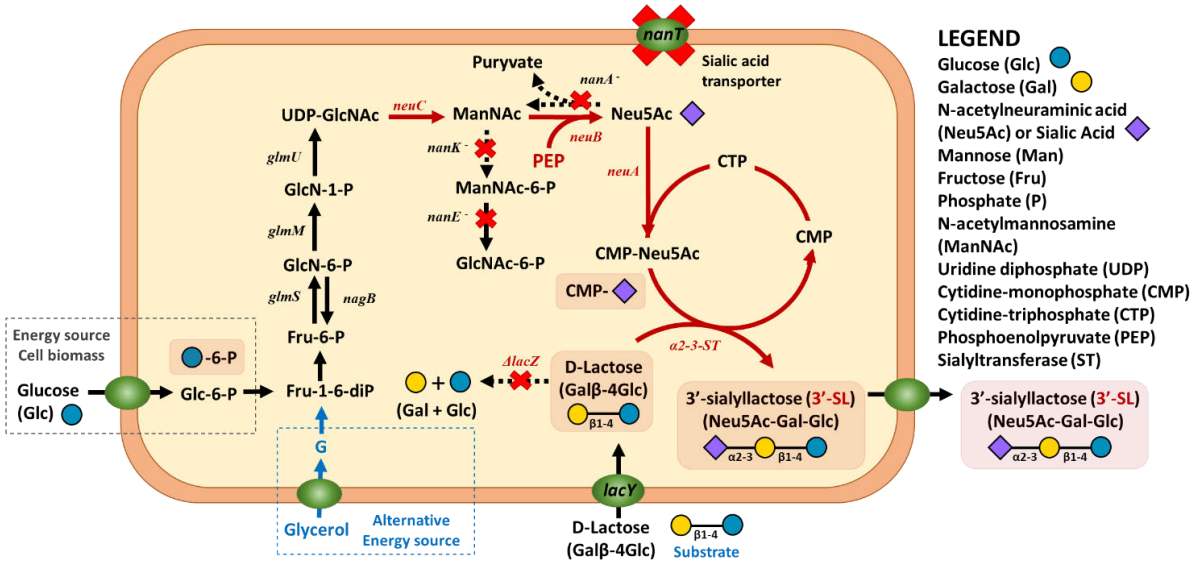


3'-SL

Similar to the 6'-SL production strain, the first modification of the 3'-SL production strain is the overexpression of a gene cluster derived from *Campylobacter jejuni* that expresses 3 enzymes involved in *de novo* synthesis of cytidine monophosphate (CMP)-sialic acid (Neu5Ac): a Neu5Ac synthase, a *N*-acetylglucosamine-6-phosphate-epimerase, and a CMP-Neu5Ac synthetase. The second modification is the overexpression of a gene derived from *Neisseria meningitides* that expresses α-2,3-sialyltransferase, an enzyme that catalyses the transfers a Neu5Ac unit from the intracellular CMP-Neu5Ac pool to the 3'-position of the D-galactose residue of D-lactose (starting substrate) in a α stereospecific manner to form the trisaccharide 3'-SL (α2,3-sialyllactose), with concurrent release of CMP.

Figure A.2.2-4 shows the biochemical pathway by which the 3'-SL production strain generates 3'-SL using D-lactose as the starting substrate and D-glucose (or alternatively D-sucrose or glycerol) as the carbon source.

Figure A.2.2-4 Engineered Pathway for 3'-SL Biosynthesis in the *E. coli* K-12 Derived Production Strain



A.3 Nature of Genetic Modification

The chromosomal genetic modifications performed on *E. coli* K-12 which led to Glycom's HiMO platform strain have previously been described in Application A1155 and evaluated by FSANZ. Genetic modifications applied to the HiMO platform strain to obtain the 2'-FL/DFL, LNT, 6'-SL, and 3'-SL production strains, as well as evidence of the stability of the genetic changes, are provided in Appendix XII **(Confidential Commercial Information)**. A brief description of the genetic modifications applied to Glycom's HiMO platform strain to generate each of the 2'-FL/DFL, LNT, 6'-SL, and 3'-SL production strains is provided below.

2'-FL/DFL

The HiMO platform strain was transformed to make the 2'-FL/DFL production strain with the aid of (i) a helper plasmid, which is responsible for expression of genes required for double strand DNA recombineering, and (ii) donor plasmids with DNA fragments including a promoter fragment, the gene(s) of interest, and a transcriptional terminator sequence flanked by sequences of homology to the site of insertion. Defined DNA sequences from donor microorganisms (such as the gene encoding alpha-1,2-fucosyltransferase from *Helicobacter pylori*) were identified using genome databanks, codon-optimised by bioinformatic tools (when needed), extended with appropriate restriction enzyme recognition sequences to allow directed cloning, and then synthesised by DNA synthesis.

The donor and helper plasmids were transformed into the HiMO platform strain *via* electroporation and removed using standardised molecular biological procedures; thus, no antibiotic resistant markers remain in

the 2'-FL/DFL production strain. The absence of the antibiotic resistant markers in the 2'-FL/DFL production strain has been confirmed by antimicrobial susceptibility testing.

The genetic modifications applied to the HiMO platform strain to generate the 2'-FL/DFL production strain were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods. The results obtained from sequencing and PCR were compared to the HiMO platform strain and confirmed that the 2'-FL/DFL production strain contains the applied genetic modifications. The 2'-FL/DFL production strain was also confirmed to be stable for over 50 generations both in terms of phenotypic performance and genotypic stability.

LNT

The HiMO platform strain was transformed to make the LNT production strain with the aid of (i) a helper plasmid, which is responsible for expression of genes required for double strand DNA recombineering, (ii) donor plasmids containing DNA fragments including a promoter fragment, the gene(s) of interest, and a transcriptional terminator sequence flanked by sequences of homology to the site of insertion, and (iii) an antibiotic marker-free plasmid for the expression of biosynthetic genes. Defined DNA sequences from donor microorganisms (such as the gene encoding beta-1,3-*N*-acetylglucosaminyltransferase from *Neisseria meningitides* and the gene encoding beta-1,3-galactosyltransferase from *Helicobacter pylori*) were identified using genome databanks, codon-optimised by bioinformatic tools (when needed), extended with appropriate restriction enzyme recognition sequences to allow directed cloning and then synthesised by DNA synthesis.

The donor and helper plasmids were transformed into the HiMO platform strain *via* electroporation and removed using standardised molecular biological procedures; thus, no antibiotic resistant markers are transferred to the production strain. One of the donor plasmids was used to incorporate a genetic cassette into the genome of the HiMO platform strain by homologous recombination to disrupt the gene cluster involved in nicotinamide adenine dinucleotide biosynthesis. The disrupted gene involved in nicotinamide adenine dinucleotide biosynthesis was then re-introduced *via* the antibiotic marker-free plasmid to tightly link cell survival to LNT production. The plasmid also contained the wild-type (non-codon-optimised) coding sequence of the beta-1,3-*N*-acetylglucosaminyltransferase gene (originating from *Neisseria meningitidis*) and the wild-type (non-codon-optimised) coding sequence of the beta-1,3-galactosyltransferase enzyme (originating from *Helicobacter pylori*). The absence of the antibiotic resistant markers in the LNT production strain has been confirmed by antimicrobial susceptibility testing.

The genetic modifications applied to the HiMO platform strain to generate the LNT production strain were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods. The results obtained from sequencing and PCR were compared to the HiMO platform strain and confirmed that the LNT production strain contains the applied genetic modifications and the episomal plasmid. The LNT production strain was also confirmed to be stable for over 50 generations both in terms of phenotypic performance and genotypic stability.

6'-SL

The HiMO platform strain was transformed to make the 6'-SL production strain with the aid of (i) a helper plasmid, which is responsible for expression of genes required for double strand DNA recombineering, (ii) donor plasmids containing DNA fragments including a promoter fragment, the gene(s) of interest, and a transcriptional terminator sequence flanked by sequences of homology to the site of insertion, and (iii) an antibiotic marker-free plasmid for the expression of biosynthetic genes. Defined DNA sequences from donor microorganisms (such as the gene encoding α-2,6-sialyltransferase from *Photobacterium damsela* and genes involved in sialic acid synthesis from *Campylobacter jejuni*) were identified using genome databanks, codon-optimised by bioinformatic tools (when needed), extended with appropriate restriction enzyme recognition sequences to allow directed cloning and then synthesised by DNA synthesis.

The donor and helper plasmids were transformed into the HiMO platform strain *via* electroporation and removed using standardised molecular biological procedures; thus, no antibiotic resistant markers are transferred to the production strain. One of the donor plasmids was used to incorporate a genetic cassette into the genome of the HiMO platform strain by homologous recombination to disrupt the gene cluster involved in nicotinamide adenine dinucleotide biosynthesis. The disrupted gene involved in nicotinamide adenine dinucleotide biosynthesis was then re-introduced *via* the antibiotic marker-free plasmid to tightly link

cell survival to 6'-SL production. This plasmid also contained an additional copy of the same bacterial gene cluster enabling synthesis of activated sialic acid that was introduced into the chromosome. The absence of the antibiotic resistant markers in the 6'-SL production strain has been confirmed by antimicrobial susceptibility testing.

The genetic modifications applied to the HiMO platform strain to generate the 6'-SL production strain were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods. The results obtained from sequencing and PCR were compared to the HiMO platform strain and confirmed that the 6'-SL production strain contains the applied genetic modifications and the episomal plasmid. The 6'-SL production strain was also confirmed to be stable for over 50 generations both in terms of phenotypic performance and genotypic stability.

3'-SL

The HiMO platform strain was transformed to make the 3'-SL production strain with the aid of (i) a helper plasmid, which is responsible for expression of genes required for double strand DNA recombineering, (ii) donor plasmids containing DNA fragments containing a promoter fragment, the gene(s) of interest, and a transcriptional terminator sequence flanked by sequences of homology to the site of insertion, and (iii) an antibiotic marker-free plasmid for the expression of biosynthetic genes. Defined DNA sequences from donor microorganisms (such as the gene encoding α-2,3-sialyltransferase from *Neisseria meningitides* and genes involved in sialic acid synthesis from *Campylobacter jejuni*) were identified using genome databanks, codon-optimised by bioinformatic tools (when needed), extended with appropriate restriction enzyme recognition sequences to allow directed cloning and then synthesised by DNA synthesis.

The donor and helper plasmids were transformed into the HiMO platform strain *via* electroporation and removed using standardised molecular biological procedures; thus, no antibiotic resistant markers are transferred to the production strain. One of the donor plasmids was used to incorporate a genetic cassette into the genome of the HiMO platform strain by homologous recombination to disrupt the gene cluster involved in nicotinamide adenine dinucleotide biosynthesis. The disrupted gene involved in nicotinamide adenine dinucleotide biosynthesis was then re-introduced *via* the antibiotic marker-free plasmid to tightly link cell survival to 3'-SL production. This plasmid also contained an additional copy of the same bacterial gene cluster enabling synthesis of activated sialic acid that was introduced into the chromosome. The absence of the antibiotic resistant markers in the 3'-SL production strain has been confirmed by antimicrobial susceptibility testing.

The genetic modifications applied to the HiMO platform strain to generate the 3'-SL production strain were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods. The results obtained from sequencing and PCR were compared to the HiMO platform strain and confirmed that the 3'-SL production strain contains the applied genetic modifications and the episomal plasmid. The production strain was confirmed to be stable for over 50 generations both in terms of phenotypic performance and genotypic stability.

B. Characterisation and Safety Assessment of New Substances

Comprehensive safety assessments of *E. coli* K-12 and its derivatives have been conducted by the U.S. EPA, from which it was concluded that *E. coli* K-12 derived strains are non-pathogenic and non-toxigenic (U.S. EPA, 1997).

The EFSA NDA Panel has previously evaluated the safety of the parental strain *E. coli* K-12 DH1, obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and deposited under DSM No. 4235 (EFSA, 2020a). Specifically, EFSA recognised that *E. coli* K-12 is a safe and non-pathogenic or toxigenic microorganism widely used for biotechnology applications, and that the whole genome of its DH1 derivative has been sequenced and concluded to be genomically different from pathogenic *E. coli* strains. The EFSA NDA Panel has also evaluated the genetic modification process applied to the parental strain to obtain Glycom's HiMO platform strain. For completeness, data on the 2'-FL/DFL, LNT, 6'-SL, and 3'-SL production strains submitted to the EFSA NDA Panel, meeting requirements of the EFSA *Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use* (EFSA, 2011), are provided in Appendix XII **(Confidential Commercial Information)**.

Notably, 2'-FL, DFL, LNT, 6'-SL, and 3'-SL are released into the fermentation broth, and intact cells of the 2'-FL/DFL, LNT, 6'-SL, and 3'-SL production strains are entirely removed from the broth without disruption by ultrafiltration after fermentation. The absence of the production strain in the final, purified 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt ingredients is confirmed by the testing of final batches against specifications for bacteria from the Enterobacteriaceae family, residual DNA (including targets for inserted DNA), residual proteins, and residual endotoxins (see Part 3.3.3, Section B.3.2, of the application).

As demonstrated by results from batch analyses (see Part 3.3.3, Section B.5.2, of the application), the purification steps involved in the manufacture of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt are proven to remove protein (*i.e.,* potential allergen) to a level of < 0.01 (w/w) %. Glycom's supplier management procedure ensures that all suppliers of raw materials and processing aids must declare and demonstrate that their materials do not contain any of the allergens specified under Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers[29].

The amino acid sequences of the recombinant proteins of the 2'-FL/DFL, LNT, 6'-SL, and 3'-SL production strains were assessed using Basic Local Alignment Search Tool (BLAST) search algorithms of Allergen Online (Version 20 for 2'-FL/DFL; Version 21 for the 3 other HiMOs) against a curated database of known and putative allergens hosted by the Food Allergen Research and Resource Program (FARRP) of the University of Nebraska (FARRP, 2021). The online tool allows search by three different search algorithms each with its own alert limit for potential allergenicity: (i) full sequence length (FASTA) comparison with an alert limit of minimum 50 % sequence similarity to hint for potential allergenic potential; (ii) 80 amino acid sequence segments (sliding window) comparison with an alert limit of minimum 35 % sequence similarity to hint for potential allergenic potential; (iii) 8 mer sequence segments (sliding window) with an alert limit of full match to hint for potential allergenic potential. No sequence alerts for potential allergenicity were identified. Therefore, it is anticipated that 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt will not pose an allergenic risk to the consumer.

C. Nutritional Impact of the Food Produced using Gene Technology

Not applicable.

D. Other Information

See Part 3.3.3 'Substances use for a Nutritive Purpose', Section C.2.1, of the application.

[29] Regulation (EU) No 1169/2011 of the European Parliament and of the Council of 25 October 2011 on the provision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004. OJ L 304, 22.11.2011, p. 18–63. Available at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32011R1169&qid=1515865791286 (current consolidated version: 01/01/2018).

PART 3.6.2 – SPECIAL PURPOSE FOOD: INFANT FORMULA PRODUCTS

A. Information Related to Composition

A.1 Purpose of Compositional Change

See Part 3.3.3 'Substances Used for a Nutritive Purpose', Section A.1, of the application.

A.2 Data for Supporting Evidence

See Section A.3 that follows.

A.3 Specific Information Requirements

A.3.1 Characterisation of Proposed Substance in Breast Milk

A.3.1.1 Human Milk Oligosaccharides

As previously discussed in Application A1155, HMOs are a complex family of structurally related oligosaccharides that form the third largest solid component in human milk after lactose and fat (Kuhn, 1952; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013). Briefly, more than 140 members of this family have been fully described on a structural basis (Urashima *et al.*, 2011; Chen, 2015), and an even higher number of members have been detected by sensitive mass spectrometry techniques (Finke *et al*., 1999; Wu *et al*., 2010, 2011). The highest concentrations of HMOs occur in human colostrum (20 to 25 g/L), and concentrations between 5 to 20 g/L are present in mature human milk (Bode, 2012). Levels of HMOs vary between individuals and are dependent on factors such as the lactation period (levels generally decrease as lactation progresses) and the genotype of the mother (*e.g.*, Lewis or Secretor genes encoding fucosyltransferases) (Castanys-Muñoz *et al.*, 2013). In contrast, bovine colostrum contains approximately 20 times lower concentrations of a far less complex oligosaccharide mixture and concentrations drop to insignificant levels at mature cow milk (Tao *et al.*, 2009; Aldredge *et al*., 2013; Urashima *et al*., 2013).

Glycom's 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt capture all 3 main types of HMOs present in human milk (*i.e.*, fucosylated, neutral core, and acidic HMOs), discussed below.

2'-FL and DFL belong to the "fucosylated" sub-fraction of HMOs, oligosaccharides that contain the sugar fucose and which is reported to constitute on average around 70 % of the total HMO fraction. In contrast, cow milk does not contain fucosylated oligosaccharides at any appreciable level (Gopal and Gill, 2000; Aldredge *et al.*, 2013). The fucosylated sub-fraction of HMOs is biosynthesised from D-lactose by specific enzymes expressed in mammary glands called the fucosyltransferases. The Secretor and Lewis status of the mother (*i.e.,* active or inactive copy of genes expressing fucosyltransfereases), categorising human milk into four different phenotypes, influences the presence and levels of fucosylated HMOs in human milk (reviewed by Castanys-Muñoz *et al*., 2013). Specifically, the biosynthesis of 2'-FL relies on the expression of α-1,2-fucosyltransferase from the FUT2 gene (Secretor phenotype). Roughly 80 % of the global population express the Secretor phenotype (Thurl *et al*., 2010; Castanys-Muñoz *et al.*, 2013; Austin *et al*., 2016).

LNT and its constitutional isomer LNnT belong to the "neutral core" sub-fraction of HMOs, which are reported to constitute approximately 15% of the total HMO fraction on average (Bode *et al*., 2012). Core HMOs like LNT and LNnT are present in all types of milks from all mother's phenotypes (Kunz *et al*., 2017). The biological relevance of the structural difference between LNT and LNnT (*i.e*., type 1 *vs*. type 2 linkage between the terminal Gal and next to last GlcNAc, and the respective ratio between the two forms) is not fully understood; however, it is equally observed in important cell-surface carbohydrate antigens like the blood group and Lewis

type antigens, and glycosphingolipids and gangliosides[30] (Yu *et al.*, 2007; Hod *et al.*, 2009). Furthermore, it had been previously observed that milk oligosaccharides of type 1 (*e.g.*, LNT) predominate over type 2 (*e.g.*, LNnT) in human milk, while the inverse is the case in all other mammalian milks (Urashima *et al.*, 2012).

6'-SL and its constitutional isomer 3'-SL belong to the acidic sub-fraction of HMOs, also called "sialylated HMOs". They are reported to constitute on average around 15 % of the total HMO fraction (Bode *et al.*, 2012). The sialylated sub-fraction of HMOs is biosynthesised from D-lactose or the "core" HMOs (HMOs that are not decorated by either L-fucose or sialic acid) by specific enzymes called sialyltransferases. Dependent on the enzyme specificity (for example α2,3- or α2,6-sialyltransferase) D-lactose can be sialylated (*i.e.*, "decorated" with sialic acid) at the 3 or 6 position of the D-galactose unit, forming 3'-SL or 6'-SL (α2,3- or α2,6-sialyllactose), respectively, the two predominant forms of sialyllactose found in milk (Martín-Sosa *et al.*, 2003; Sprenger *et al.*, 2017).

A.3.1.2 Levels in Human Breast Milk

A published systematic review by Thurl *et al.* (2017) presenting a meta-analysis of the concentrations of oligosaccharides in human milk reported mean values for a number of HMOs in milk from Secretor mothers who delivered term or preterm infants. Overall, 21 studies were determined to meet predefined inclusion criteria. Reported mean concentrations and 95 % confidence limits for 2'-FL, DFL, LNT, 6'-SL, and 3'-SL are reproduced in Table A.3.1.2-1. Generally, mean concentrations were higher in Secretor milk collected within the first month postpartum from mothers who delivered preterm infants compared to Secretor milk collected over a longer duration postpartum (up to 100 lactation days) from mothers who delivered term infants.

Table A.3.1.2-1 Mean Concentrations of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL from Human Milk of Secretor Mothers (Adapted from Thurl *et al.*, 2017)

	Term Infants (0 to 100 Lactations Days)			Preterm Infants (0 to 60 Lactation Days)		
HMO	**No. Studies**	**Mean**	**95 % CL**	**No. Studies**	**Mean**	**95 % CL**
2'-FL (g/L)	10	2.74	2.43 to 3.04	3	2.77	0.76 to 4.78
DFL (g/L)	6	0.42	0.32 to 0.51	2	0.41	0.17 to 0.65
LNT (g/L)	8	0.79	0.59 to 0.98	3	1.04	0.39 to 1.68
6'-SL (g/L)	6	0.64	0.38 to 0.91	1	0.66*	0.25 to 1.08*
3'-SL (g/L)	4	0.19	0.14 to 0.24	2	0.29*	0.21 to 0.36*

2'-FL = 2'-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; CL = confidence limit; DFL = difucosyllactose; HMO = human milk oligosaccharide; LNT = lacto-*N*-tetraose.
* 0 to 30 lactation days

More recently, Soyyılmaz *et al.* (2021) conducted a systematic analysis of global pooled (Secretor and non-Secretor) mean concentrations of individual HMOs in human milk from healthy mothers during pre-defined lactation periods. Overall, 57 peer reviewed articles published between 1996 and 2020 and conducted in sample populations from 31 countries were identified as reporting mean concentrations of individual HMOs. Mean HMO concentrations reported according to Secretor status were converted to a pooled level-based on the typically observed frequency of Secretor/non-Secretor phenotypes in the global population (80/20 %, respectively). Analyses were conducted using a weighted-average accounting for sample size. The resulting mean concentrations of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL in colostrum (0 to 5 days), transitional milk (6 to 14 days), mature milk (15 to 90 days), and late milk (> 90 days) are summarised in Table A.3.1.2-2. A limitation of this study is that only the mean of means and the range of means are reported, with no indication of the full range or statistical distribution (*i.e.*, percentile confidence levels) of HMO concentrations in human milk. Furthermore, analyses considered HMO levels from pooled milk (rather than Secretor versus non-Secretor milk) as the objective of the study was to provide representative concentrations of individual HMOs on a

[30] The linkage difference between LNT and LNnT (*i.e.*, Gal-ß(**1**-**3**)-GlcNAc *vs.* Gal-ß(**1**-**4**)-GlcNAc) is for historical reasons called "type 1" and "type 2" in blood group and Lewis antigen research, while the identical structural difference is called "lacto" and "neolacto" series in glycolipid research.

global level regardless of individual variations. As such, reported mean concentrations from pooled milk do not represent the full distribution of HMO concentrations naturally occurring in human milk.

Table A.3.1.2-2 Global Pooled Concentrations of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL in Human Milk by Lactation Period (Adapted from Soyyılmaz *et al.*, 2021)

HMO	Colostrum (0 to 5 d)	Transitional (6 to 14 d)	Mature (15 to 90 d)	Late (> 90 d)
2'-FL (g/L)	Mean of means (range) 3.18 (0.69 to 4.28) Median 2.30	Mean of means (range) 2.07 (0.10 to 2.88) Median 2.60	Mean of means (range) 2.28 (0.69 to 4.28) Median 2.30	Mean of means (range) 1.65 (0.00 to 4.27) Median 1.72
DFL (g/L)	Mean of means (range) 0.29 (0.04 to 0.54) Median 0.32	Mean of means (range) 0.56 (0.40 to 0.7) Median 0.68	Mean of means (range) 0.29 (0.04 to 0.54) Median 0.32	Mean of means (range) 0.27 (0.00 to 0.58) Median 0.27
LNT (g/L)	Mean of means (range) 0.73 (0.20 to 1.60) Median 0.62	Mean of means (range) 1.07 (0.36 to 3.9) Median 0.88	Mean of means (range) 0.74 (0.2 to 1.60) Median 0.62	Mean of means (range) 0.64 (0.10 to 1.37) Median 0.56
6'-SL (g/L)	Mean of means (range) 0.40 (0.00 to 0.74) Median 0.45	Mean of means (range) 0.71 (0.00 to 1.30) Median 0.73	Mean of means (range) 0.40 (0.00 to 0.74) Median 0.45	Mean of means (range) 0.30 (0.01 to 1.00) Median 0.19
3'-SL (g/L)	Mean of means (range) 0.19 (0.00 to 0.67) Median 0.14	Mean of means (range) 0.13 (0.00 to 0.25) Median 0.13	Mean of means (range) 0.19 (0.00 to 0.7) Median 0.14	Mean of means (range) 0.13 (0.08 to 0.30) Median 0.13

2'-FL = 2'-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; d = days; DFL = difucosyllactose; HMO = human milk oligosaccharide; LNT = lacto-*N*-tetraose.

Maximum proposed use levels of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt for use in infant formula products were selected to reflect **mean** concentrations of the corresponding HMOs that are naturally present in human milk (see Part 3.3.3, Section D.2, of the application). Accordingly, the estimated exposure to 2'-FL, DFL, LNT, 6'-SL, and 3'-SL from its proposed use in infant formula products will be comparable to the intakes of these HMOs by breastfed infants (see Part 3.3.3, Section D.3.3, of the application).

A.3.1.3 Estimated Daily Intake from Human Breast Milk

FSANZ Estimates

In their evaluation of Application A1155, FSANZ estimated the daily intakes of 2'-FL from human milk in infants 3 months and 9 months of age based on mean reported concentrations in mature milk (from Secretor milk samples) and infant energy requirements (FSANZ, 2019c). 2'-FL intake estimates and default values are reproduced in Table A.3.1.3-1.

Table A.3.1.3-1 Calculation of Estimated Dietary Intakes of 2'-FL from Human Milk for Infants Aged 3 Months and 9 Months (Reproduced from FSANZ, 2019c)

	Units	10 to 60 Day Secretor Human Milk	60+ Days Secretor Human Milk	
		3 Months	3 Months	9 Months
Recommended energy intake[a]	kJ/kg bw/day	343	343	330
P50 Body Weight[b]	kg	6.4	6.4	8.9
Recommended energy intake	kJ/day	2,195	2,195	2,937
Amount of human milk required to meet energy requirements[c]	g/day	765	765	n/a
Amount of human milk required to meet 50 % of energy requirements[c]	g/day	n/a	n/a	515
Mean dietary intake of 2'-FL_{human}[d]	g/day	2.2	1.8	1.2
	g/kg bw/day	0.34	0.28	0.13
P90 dietary intake of 2'- FL_{human}[d,e]	g/day	4.4	3.5	2.4
	g/kg bw/day	0.69	0.55	0.27

2'-FL = 2'-fucosylactose; bw = body weight; n/a = not available; P50 = 50th percentile; P90 = 90th percentile.

[a] (FAO/WHO/UNU, 2004)

[b] (WHO, 2006)

[c] Energy content of human milk is 286 kJ/100 g (Food Standards Australia New Zealand 2016 – FSANZ, 2016). One litre of human milk is equivalent to 1,040 grams.

[d] Mean concentration of 2'-FL in human milk, from Secretor milk samples, used in calculation of 3.0 g/L 10 to 60 days postpartum and 2.4 g/L 60+ days postpartum.

[e] Dietary intakes at the 90th percentile were calculated by doubling the mean intake value.

Dietary intakes of DFL, LNT, 6'-SL, and 3'-SL from human milk were estimated using the same default values for infant energy requirements, and by applying the highest mean concentration of the HMO from Secretor milk calculated in the meta-analysis by Thurl *et al*. (2017). High-level dietary intakes at the 90th percentile were estimated by doubling the mean exposure. The resulting mean and 90th percentile estimated daily intakes of DFL, LNT, 6'-SL, and 3'-SL from human milk are presented in Table A.3.1.3-2.

Table A.3.1.3-2 Calculation of Estimated Dietary Intakes of DFL, LNT, 6'-SL, and 3'-SL from Secretor Human Milk for Infants Aged 3 Months and 9 Months[a]

HMO	Highest Mean Concentration from Thurl *et al.* (2017)	Statistic	Units	Dietary Intake from Secretor Human Milk	
				3 Months	9 Months
DFL	0.42 g/L	Mean	g/day	0.31	0.21
			g/kg bw/day	0.05	0.02
		P90[b]	g/day	0.62	0.42
			g/kg bw/day	0.10	0.05
LNT	1.04 g/L	Mean	g/day	0.77	0.52
			g/kg bw/day	0.12	0.06
		P90[b]	g/day	1.5	1.03
			g/kg bw/day	0.24	0.12
6'-SL	0.66 g/L	Mean	g/day	0.49	0.33
			g/kg bw/day	0.08	0.04
		P90[b]	g/day	0.97	0.65
			g/kg bw/day	0.15	0.07
3'-SL	0.29 g/L	Mean	g/day	0.21	0.14
			g/kg bw/day	0.03	0.02
		P90[b]	g/day	0.43	0.29
			g/kg bw/day	0.07	0.03

3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; bw = body weight; DFL = difucosyllactose; HMO = human milk oligosaccharide; LNT = lacto-*N*-tetraose; P90 = 90th percentile.

[a] Calculated based on default values for infant energy requirements previously used by FSANZ (2019c).

[b] Dietary intakes at the 90th percentile were calculated by doubling the mean intake value.

EFSA Estimates

In their evaluations of the safety of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt following requests to the European Commission by Glycom, the European Food Safety Authority (EFSA) Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel) estimated the daily intake levels of the relevant HMOs from human milk (EFSA, 2019a,b, 2020a,b, 2022). Estimates were based on mean and highest occurrence levels of 2'-FL, DFL, and LNT from pooled human milk during different lactating stages reported by Erney *et al.* (2001) or on highest mean and upper confidence limit of the mean levels of 6'-SL and 3'-SL from milk of mothers who delivered preterm summarised by Thurl *et al.* (2017). EFSA has also since evaluated the safety of LNT and 3'-SL sodium salt produced by derivative strains of *E. coli* BL21 (DE3) as novel foods (EFSA, 2022b,c). Estimated daily intakes of LNT from human milk were again based on mean and high concentrations reported by Erney *et al.* (2001) and thus remain unchanged, whereas estimated daily intakes of 3'-SL were based on mean of mean and maximum mean concentrations reported by Soyyılmaz *et al.* (2021) for mature milk. In all cases, the calculations also took into consideration average (800 mL) and high (1,200 mL) daily intake levels of human milk by infants up to 6 months of age (EFSA, 2013), and a default body weight of 6.7 kg for infants 3 to 6 months of age who are likely to consume these volumes of human milk (EFSA, 2012a). EFSA's most recent estimated daily intake levels of 2'-FL/DFL, LNT, 6'-SL, and 3'-SL are reproduced in Table A.3.1.3-3 below.

The range of high level (90th percentile) dietary intakes of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL from human milk calculated using the FSANZ infant energy requirements approach (Tables A.3.1.3-1 and A.3.1.3-2) are generally within the range of EFSA-reported estimated daily intakes for these HMOs calculated on a consumption basis (Tables A.3.1.3-3).

Table A.3.1.3-3 Estimated Daily Intake Levels of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL from Human Milk for Infants of 6.7 kg bw (Adapted from EFSA, 2019a,b, 2020a, 2022c)

HMO	Concentration	Average Daily Intake Levels (mg/kg bw) from 800 mL of Human Milk	High Daily Intake Levels (mg/kg bw) from 1,200 mL of Human Milk
2'-FL	2.38 g/L (mean)[a]	284	426
	4.78 g/L (max)[a]	571	856
DFL	0.46 g/L (mean)[a]	55	82
	2.44 g/L (max)[a]	291	437
LNT	0.76 g/L (mean)[a]	91	136
	2.74 g/L (max)[a]	327	491
6'-SL	0.66 g/L (mean)	79	118
	1.08 g/L (max)	129	193
3'-SL	0.19 g/L (mean)[c]	23	84
	0.70 g/L (max)[c]	34	125

2'-FL = 2'-fucosyllactose; 3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; bw = body weight; DFL = difucosyllactose; HMO = human milk oligosaccharide; LNT = lacto-*N*-tetraose; Max = maximum.
[a] Reported by Erney *et al.* (2001).
[b] Reported by Thurl *et al.* (2017).
[c] Reported by Soyyılmaz *et al.* (2021).

A.3.2 Nutritional Safety and Tolerance

See Part 3.3.3 'Substances Used for a Nutritive Purpose' of the application, Section C.2.2.

A.3.3 Efficacy of Proposed Compositional Change

See Part 3.3.3 'Substances Used for a Nutritive Purpose' of the application, Section A.2.

B. Dietary Intake or Dietary Exposure

B.1 Dietary Intake or Exposure of Target Population

Estimated daily intakes of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL from the intended conditions of use of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt in infant formula products is presented in Part 3.3.3, Section D.3, of the application. These are compared below to estimated daily intakes to corresponding HMOs in human milk (discussed in Section A.3.1.3).

FSANZ previously concluded that dietary intakes of 2'-FL from use in infant formula products are similar to those from human milk in infants 3 and 9 months of age, as the maximum use level of 2'-FL in infant formula and follow-on formula (96 mg/100 KJ or 2.4 g/L) is similar to the mean concentration of 2'-FL from Secretor milk (FSANZ, 2019c). As 2'-FL/DFL is intended to be used as an alternative source of the already authorised 2'-FL in infant formula products (*i.e.*, 2'-FL alone or in combination with LNnT at up to 96 mg/100 KJ), intake estimates previously summarised for 2'-FL by FSANZ remain relevant to the current application and are reproduced in Table B.1-1.

Table B.1-1 Summary of Estimated Dietary Intakes of 2'-FL (from All Sources Assessed) for Infants 3 Months and 9 Months of Age (Reproduced from FSANZ, 2019c)

Substance	Unit	Age Group	Mean Dietary Intake		P90 Dietary Intake	
			From Microbial Fermentation	From Human Milk	From Microbial Fermentation	From Human Milk
2'-FL	g/day	3 months	2.1	1.8 to 2.2[a]	4.2	3.5 to 4.4[a]
		9 months	1.4	1.2	2.8	2.4
	g/kg bw/day	3 months	0.33	0.28 to 0.34[a]	0.66	0.55 to 0.69[a]
		9 months	0.16	0.13	0.32	0.27

2'-FL = 2'-fucosyllactose; bw = body weight; P90 = 90th percentile.
[a] Lower bound of the range is for human milk 60+ days post-partum; upper bound of the range if for 10-60 days post-partum.

Taking into consideration infant energy requirements similar to the FSANZ approach for 2'-FL, Table B.1-2 compares estimated daily intakes of DFL, LNT, 6'-SL, and 3'-SL under the maximum proposed use levels in infant formula products (taken from Part 3.3.3, Table D.3.1-2, of the application) to intake estimates from naturally occurring levels of the corresponding HMOs in human milk (taken from Table A.3.1.3-2 of this part of the application).

Table B.1-2 Comparison of DFL, LNT, 6'-SL, and 3'-SL Estimated Daily Intakes from Human Milk to Infant Formula Under the Proposed Conditions of Use

HiMO	Unit	Age Group	Mean Dietary Intake		P90 Dietary Intake	
			From Microbial Fermentation[a]	From Human Milk[b]	From Microbial Fermentation[a]	From Human Milk[b]
DFL	g/day	3 months	0.25	0.29	0.50	0.59
		9 months	0.17	0.20	0.34	0.40
	g/kg bw/day	3 months	0.04	0.05	0.08	0.09
		9 months	0.02	0.02	0.04	0.04
LNT	g/day	3 months	0.70	0.77	1.4	1.53
		9 months	0.47	0.52	0.94	1.03
	g/kg bw/day	3 months	0.11	0.12	0.22	0.24
		9 months	0.05	0.06	0.11	0.12
6'-SL	g/day	3 months	0.35	0.49	0.70	0.97
		9 months	0.24	0.33	0.47	0.65
	g/kg bw/day	3 months	0.05	0.08	0.11	0.15
		9 months	0.05	0.04	0.05	0.07
3'-SL	g/day	3 months	0.18	0.21	0.35	0.43
		9 months	0.12	0.14	0.24	0.29
	g/kg bw/day	3 months	0.03	0.03	0.05	0.07
		9 months	0.01	0.02	0.03	0.03

3'-SL = 3'-sialyllactose; 6'-SL = 6'-sialyllactose; bw = body weight; DFL = difucosyllactose; HiMO = human-identical milk oligosaccharide; LNT = lacto-*N*-tetraose; P90 = 90th percentile.
[a] Taken from Table D.3.1-2.
[b] Taken from Table A.3.1.3-2 of Part 3.6.2

Estimated dietary intakes of DFL, LNT, 6'-SL, and 3'-SL from maximum proposed levels of use of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt in infant formula products are within dietary intake estimates of these same naturally occurring HMOs from human milk. This is due to maximum proposed use levels of 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt in infant formula products, expressed on a HiMO basis, being similar to the mean concentration of corresponding HMOs in human milk.

B.2 Level of Formula Consumption

Not applicable.

B.3 Information Relating to the Substance

The history of safe consumption of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL as natural components in human breast milk is discussed in Section A of this part of the application. HMOs are not present in mature cow milk (or any other farm animal milk) to any significant degree (Urashima *et al*., 2013; Albrecht *et al*., 2014; Wang *et al*., 2020). Sialylated oligosaccharides account for the majority of the oligosaccharide fraction in bovine milk, though levels are more than 50 times lower than those in human milk (Aldredge *et al*., 2013; Urashima *et al*., 2013; Albrecht *et al*., 2014 – taken from EFSA, 2020a). Therefore, there is likely to be minimal contribution from these sources and their by-products to total dietary intakes of 2'-FL, DFL, LNT, 6'-SL, and 3'-SL in infants.

C. Labelling Requirements under Part 2.9 of the Code

C.1 Safety or Nutritional Impact of Labelling Change

See Part 3.3.3 'Substances Used for a Nutritive Purpose', Section B.7, of the application.

C.2 Demonstrated Consumer Understanding of Labelling Change

See Part 3.3.3 'Substances Used for a Nutritive Purpose', Section G.1, of the application.

D. Internationally Recognised Standards, Codes of Practice, Recommendations and Guidelines on Labelling

In addition to the general labelling requirements established under Part 1.2 of the Code, infant formula products containing 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt will be labelled in accordance with the specific provisions established in Standard 2.9.1 of the Code.

International labelling standards that are applicable to infant formula products, including those containing 2'-FL/DFL, LNT, 6'-SL sodium salt, and 3'-SL sodium salt alone or in combinations, include labelling standards from the International Code of Marketing of Breastmilk Substitutes (WHO, 1981) and the following Codex Alimentarius guidelines and standards on general labelling of foods:

- Guidelines on Nutrition Labelling (Codex Alimentarius, 2021);
- Guidelines on Formulated Complementary Foods for Older Infants and Young Children (Codex Alimentarius, 2017);
- General Standard for the Labelling of Prepackaged Foods (Codex Alimentarius, 2018); and
- General Standard for the Labelling of and Claims for Prepackaged Foods for Special Dietary Uses (Codex Alimentarius, 2009).

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