Submitted to
Food Standards Australia New Zealand (FSANZ)

Application to Amend the Australia New Zealand Food Standards Code:

2'-Fucosyllactose (2'-FL), 3-Fucosyllactose (3-FL), Lacto-*N*-tetraose (LNT), 3'-Sialyllactose (3'-SL) sodium salt, and 6'-Sialyllactose (6'-SL) sodium salt for Use as Nutritive Substances in Infant Formula Products.

Prepared by Chr. Hansen A/S (Part of Novonesis Group)

V5 (Amended 25 November 2025)

Statutory Declaration

I, Chandrika Balachandrun, Head of HHB RA APAC, B-15-3, Level 15, The Ascent, Paradigm No, 1, Jalan SS 7/26a, 47301 Petaling Jaya, Selangor, Malaysia, make the following declaration under section 9 of the *Statutory Declarations Act 1959*:

- The information provided in this application fully sets out the matters required.
- The information provided in this application is true to the best of my knowledge and belief.
- No information has been withheld that might prejudice this application, to the best of my knowledge and belief.

I believe that the statements in this declaration are true in every particular, and I understand that a person who intentionally makes a false statement in a statutory declaration is guilty of an offence under section 11 of the Statutory Declarations Act 1959, the punishment for which is imprisonment for a term of 4 years.

[redacted] Head of HHB RA APAC

[redacted]

Declared at B-15-3, Level 15, The Ascent, Paradigm No, 1, Jalan SS 7/26a, 47301 Petaling Jaya, Selangor, Malaysia on 24 of July, 2025.

Observed by me,

[redacted], B-15-3, Level 15, The Ascent, Paradigm No, 1, Jalan SS 7/26a, 47301 Petaling Jaya, Selangor, Malaysia

[redacted]

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Checklists

General requirements (3.1.1)		
Check	**Page No.**	**Mandatory Requirements**
☑	N/A	A Form of application ☑ Application in English ☑ Executive Summary (separated from main application electronically) ☑ Relevant sections of Part 3 clearly identified ☑ Pages sequentially numbered ☑ Electronic copy (searchable) ☑ All references provided
☑	12	B Applicant details
☑	13	C Purpose of the application
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☑	18	L Checklist/s provided with application ☑ 3.1.1 Checklist ☑ All page number references from application included ☑ Any other relevant checklists for Chapters 3.2–3.7

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☐	N/A	B.4 Novel herbicide metabolites in GM herbicide-tolerant plants
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3.1.1 General Requirements

B. Applicant Details

Applicant Name

Chr. Hansen A/S

Contact Person(s) and Details

[redacted]

Address

Notifier address
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Denmark

Local Address
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North Rocks
NSW 2151
Australia

Nature of Applicant's Business

As of 29 January 2024, the combination of two companies, **Chr. Hansen A/S** and **Novozymes A/S**, was closed. As such, Chr. Hansen A/S, Novozymes A/S, and subsidiaries are now part of the **Novonesis** group. The official statement regarding this name change is attached as Appendix 01a (**COMMERCIALLY CONFIDENTIAL INFORMATION**).

Novonesis is a global biosolutions company with a diverse portfolio, specialising in the development of natural ingredient solutions. The company operates through two main divisions. The Food and Health Biosolutions division focuses on enhancing food quality and promoting human health through innovations such as food cultures, pharmaceuticals, probiotics, and human milk oligosacharrides (HMOs). Meanwhile, the Planetary Health Biosolutions division emphasizes sustainable practices in agriculture and household care, aiming to reduce chemical usage and support climate-neutral initiatives.

Chr. Hansen acquired **Jennewein Biotechnologie GmbH** as of 09 October 2020, following which Jennewein Biotechnologie GmbH underwent a name change to **Chr. Hansen HMO GmbH** effective 04

May 2021. The official statement regarding this name change is attached as Appendix 01b (**COMMERCIALLY CONFIDENTIAL INFORMATION**).

In August 2019, Jennewein Biotechnologie GmbH had made an application (A1190) to FSANZ for permission to use 2'-FL as a food ingredient in infant formula and toddler formula. The approval report as published by FSANZ on 8 November 2021 permitted the voluntary addition of 2'-FL in infant formula products (IFP) and formulated supplementary foods for young children (FSFYC).

Details of other individuals, companies, or organisations associated with the application

The preparation, submission, and stewardship of this application was handled in its entirety by Novonesis.

C. Purpose of Application

Chr. Hansen A/S (Chr. Hansen) is seeking permission to amend Schedule 3 (Identity and Purity) and Schedule 26 (Food Produced using Gene Technology) of the Australia New Zealand Food Standards (FSANZ) Code (the Food Standards Code) to include Chr. Hansen's 2'-Fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-*N*-tetraose (LNT), 3'-sialyllactose sodium salt (hereafter referred to as 3'-SL), and 6'-sialyllactose sodium salt (hereafter referred to as 6'-SL) for use as nutritive substances alone and/or in combination, in infant formula products (including infant formula, follow-on formula, and Special Medical Purpose Products for Infants (SMPPi)).

The combination of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL has been trademarked as MyOli™, but is referred to in this application as 5HMO-Mix.

Chr. Hansen intends to use 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL as ingredients for use in infant formula products to better reflect the compositional profile of HMOs present in human milk.

D. Justification for the Application

Human breast milk contains a unique fraction of structurally diverse non-digestible carbohydrates known as human milk oligosaccharides (HMOs), which represent the third most abundant solid component after lactose and lipids. While breastfeeding is the preferred method of infant nutrition as supported by numerous organisations and governmental bodies including those in Australia, this form of infant nutrition may not always be a viable option for all infants and mothers. For infants under 12 months of age, commercially available infant formulas are considered to be the safest alternative to human breast milk (NHMRC, 2012). It is therefore important that these commercially available infant formula products more closely match the nutrition composition of human breast milk. This is due to the fact that while oligosaccharides are a large component of human breast milk, this is not the case for milk from bovine sources, which is the type of milk most commonly used in infant formula products.

As shown in this application, Chr. Hansen's HMOs (2'-FL, 3-FL, LNT, 3'-SL, 6'-SL used alone or in combination) are chemically and structurally identical to those that are naturally occurring in human

breast milk. Additionally, information on the benefits and history of consumption of these HMOs is also presented in this application.

Information showing that the production organisms used to produce Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL lack the genes necessary for invasion, adhesion, as well as lacking the enterotoxins necessary for pathogenicity has also been provided as part of this application. These HMO ingredients are also subject to a purification process to eliminate the production organism (**Section 3.3.3 D.5**).

In addition to this, Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL has been approved for addition (either singly or in combination) into infant formula product equivalents and more in numerous countries including Europe, the United States, and Canada among others (**Section 3.3.3 C.3** and Appendix 02 (**COMMERCIALLY CONFIDENTIAL INFORMATION**)).

As such, it is anticipated that the approval of Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL (used alone or in combination) as food produced using gene technology and a nutritive substance in infant formula products will benefit consumers as well as industry in Australia by providing high quality infant formula products that are more closely aligned with the composition of human breast milk.

D.1 Regulatory Impact Information

D.1.1 Costs and benefits of the application

As food technology advances, manufacturers are increasingly adding new substances to infant formula to better reflect the compositional profiles of oligosaccharides present in human breast milk. This is aligned with the purpose of adding Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL to these product categories.

Consumers are most likely to benefit due to the availability of such formula which provides additional nutritional components. Additional information on the purpose and health benefits of from the consumption of formula containing 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are described in **Section 3.3.3 A.1**.

With regards to the industry, formula manufacturers are likely to benefit from having a wider variety of sources for HMOs, which in turn may increase the overall growth of the product category as well as innovation and competition between formula manufacturers. This will in turn benefit the consumer in terms of cost and provide additional, more nutritional product choices to consumers in Australia.

As indicated in **Section 3.1.1 F**, Chr. Hansen intends to pay the full cost of processing this application to amend the Food Standards Code. As such, there is no expected cost to the Australian government with regards to this application. Additionally, the approval of use of these HMOs in Australia may also have potential economic gains to Australia as a result of increased innovation with regards to infant formula products.

D.1.2 Impact on international trade

At present, Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are permitted for use in infant formula, follow-up formula, formulated milk powder for children, and in food supplements. These HMOs have

also been granted GRAS status by the US FDA, and approval for usage in Singapore, Canada, and India. The approval letters for use of these HMOs in Canada and India are provided in Appendix 02 (**COMMERCIALLY CONFIDENTIAL INFORMATION**) and Appendix 03 (**COMMERCIALLY CONFIDENTIAL INFORMATION**) respectively. 2'-FL is also approved for use in Malaysia.

Additionally, these HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) are included on the Therapeutic Goods (Permissible Ingredients) Determination in Australia for use as complementary medicine ingredients in listed medicines in Australia.

Other beneficial impacts to trade would include that those manufacturers presently using Chr. Hansen's HMOs would be able to expand into Australia as a result of this approval, as this would eliminate the trade barrier resulting from the use of these ingredients. It is also understood that a number of dairy companies in China have invested in food manufacturing facilities (also producing infant formula), which will contribute to positive impact on international trade for Australia.

As such, it is anticipated that the approval of Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL by FSANZ would have a positive impact on international trade.

E. Information to support the application

E.1 Data requirements

Based on information and requirements as presented in the FSANZ Application Handbook, Chr. Hansen has provided information to meet the requirements as specified in the following guidelines from the Handbook:

- 3.1.1 General Requirements
- 3.3.3 Substances used for a Nutritive Purpose
- 3.5.1 Foods Produced using Gene Technology
- 3.6.2 Special Purpose Food – Infant Formula Products

In addition to this, Chr. Hansen has also provided information as listed in the List of Appendices on **Page 8**.

A literature search was carried out to provide supporting information for this application. The search was carried out using the United States National Library of Medicine National Institutes of Health Pubmed database in July 2025.

A total of 383 publications were identified from the searches carried out.

Further details of the literature search, including the terms used, are provided in Appendix 04. Copies of the publications used to support this application are provided in the folder "**Chr. Hansen Application - References**" which has been provided as part of this application.

F. Assessment Procedure

Based on the FSANZ Application Handbook and past application A1190, Chr. Hansen proposes that this Application would likely be considered under the '**General Procedure**' category.

G. Confidential Commercial Information (CCI)

Chr. Hansen would like to keep the content of the appendices provided as part of this application confidential, as listed in Table 1.

Table 1: Appendices indicated to contain Confidential Commercial Information (CCI)

Appendix	Name
Appendix 01a	Chr Hansen Novozymes Name Change
Appendix 01b	Jennewein Chr Hansen Name Change
Appendix 02	Health Canada LONO
Appendix 03	India Form II (Approval Letter)
Appendix 07	NMR and MS Analyses
Appendix 08	Details of the Production Strains
Appendix 09	Stability of the Individual HMOs
Appendix 10	Stability Study Report
Appendix 11	Internal Test Methods
Appendix 12	qPCR Validation and Test Reports
Appendix 13	Absence of Viable Cells
Appendix 14	FSSC CH HMO 2024-25
Appendix 15	Certifications of Manufacturing Facilities and Testing Labs
Appendix 16	Certificates of Analysis
Appendix 17	Reports for Toxicological Studies
Appendix 18	Stability of 5HMO-Mix in Infant Formulae
Appendix 23	Strain Stability Reports
Appendix 24	DNA and Amino Acid Sequences

The information contained in the appendices as included as part of this application are considered to be CCI as defined by the FSANZ Act 1991, as Chr. Hansen has invested significant time and financial resources to develop the technology to manufacture 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL substances.

H. Other Confidential Information

Not applicable.

I. Exclusive Capturable Commercial Benefit (ECCB)

Chr. Hansen is seeking exclusive permission for the use of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL (alone and/or in combination) produced using technology on the basis that they are highly refined products obtained via a proprietary manufacturing process. Chr. Hansen and partners have also carried out significant research and investment with regards to the development of these ingredient.

It is anticipated that the exclusivity will be specific to Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL when they are used as ingredients, and not to finished products containing the material. In practice, this indicates that during the exclusivity period, a manufacturer may incorporate 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL alone and/or in combination into infant formula products only if they have obtained the ingredients from Chr. Hansen with the intention to use these ingredients in accordance with the agreed conditions as provided in the approval from FSANZ.

As such, this application, if approved, is likely to result in an amendment to the Food Standards Code that provides Exclusive Capturable Commercial Benefit (ECCB) to Chr. Hansen. Therefore, Chr. Hansen intends to pay the full cost of processing this application.

J. International and Other National Standards

J.1 International Standards

The Codex Alimentarius Commission Standards relevant to this application include:

- *CXS 72-1981 Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants. Revision 2007.*
- *CXS 156-1987 Standard for Follow-Up Formula. Revision 2023.*
- *CAC/GL 8-1991 Guidelines on Formulated Complementary Foods for Older Infants and Young Children. Revision 2017.*

J.2 Other National Standards or Regulations

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

- **EU:** European Commission (EC) Directive 2006/141/EC (EC Directive, 2006)
- **United States:** Federal Food and Drug and Cosmetic Act (FFDCA) Section 412 and US FDA's Code of Federal Regulations (21 CFR) Title 21.
- **China:** Administrative Measures for the Registration of Recipes for Formula Powder Products for Infants and Young Children (CFDA Decree 26)
- **Malaysia:** Malaysia Food Regulation Act 1985 Regulation 91B regulates 'Formulated milk powder for children'. This covers children aged 12 months to 9 years.
- **Singapore:** Food Regulations, Sale of Food Act. Regulation 252 – Infant Formula.

K. Statutory Declaration

A signed statutory declaration is provided on **Page 2** of this application.

L. Checklist

The relevant completed checklists are provided on **Page 9** of this application.

3.3.3 Substances Used for a Nutritive Purpose

A. Information on the use of the nutritive substance

A.1 Information on the purpose of the use of a nutritive substance in food

Introduction

Humans are exposed to fucosylated oligosaccharides such as 2'-FL and 3-FL (3-fucosyllactose) as well as other HMOs such as LNT, 3'-SL, and 6'-SL during the early years of life especially while nursing as infants. Statements provided by the Australian National Health and Medical Research Council (NHMRC) indicate breastfeeding is important for the nutrition, immunological protection, growth, and development of infants and toddlers, and notes breastfeeding is the normal and unequalled method of feeding infants (NHMRC, 2012). The same group recommends breastfeeding until 12 months of age and to continue as long past one year of age as the mother and child wish (NHMRC, 2012). The World Health Organization (WHO) and United Nations Children's Fund (UNICEF) recommend exclusive breastfeeding for the first six months of age and continued breastfeeding with complementary foods up to two years of age (WHO/UNICEF, 2003).

Human milk is recommended as the first food for infants because it provides optimum nutrition and immunity benefits and reduces instances of disease later in life such as asthma, type 1 diabetes, and childhood leukaemia (Agostoni et al., 2009; Field, 2005; Kunz et al., 1999; van Rossum et al., 2005). Many epidemiological studies also suggest that infants who are exclusively breastfed have improved cognition later in childhood (Deoni et al., 2013). Other studies indicate that breastfeeding influences the gene expression profile in the infant intestine, and it is likely that dietary factors provided by the mother's milk contribute to this differential gene expression (Chapkin et al., 2010).

Human milk contains all essential nutrients for infants, including proteins, essential fatty acids, carbohydrates (including HMOs), minerals, vitamins and trace elements, in addition to immunity-related and developmental components such as IgA, leucocytes, oligosaccharides, lysozyme, lactoferrin, interferon-γ, nucleotides, cytokines, growth factors, hormones and other biologically active molecules (Field, 2005; Kunz et al., 1999).

Human Milk Oligosaccharides

Human milk oligosaccharides (HMOs) represent the third largest component of human milk after lactose and total lipids (Bode, 2012, 2019). Oligosaccharides in human milk are structurally diverse compounds consisting of monosaccharide building blocks, specifically glucose (Glc), galactose (Gal), N-acetylglucosamine (GlcNAc), fucose (Fuc), and N-acetylneuraminic acid (Neu5Ac), that are connected by various glycosidic linkages (Bode, 2019; Walsh et al., 2020).

HMO synthesis occurs in the lactating mammary gland under the control of a series of glycosyltransferases, including fucosyltransferases, sialyltransferases, galactotransferases, and acetylglucosaminyltransferases (Bode, 2019; Walsh et al., 2020). The most predominant HMOs include 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-*N*-tetraose (LNT), 3'-sialyllactose (3'-SL) and 6'-sialyllactose (6'-SL). Although HMOs are diverse in structure, they can be assigned to three main classes:

- neutral fucosylated HMOs (containing fucose), such as 2'-FL and 3-FL
- neutral core HMOs (containing the amino sugar GlcNAc), such as LNT
- acidic HMOs (containing sialic acid), such as 3'-SL and 6'-SL.

All HMOs carry lactose (Galβ1–4Glc) at the reducing end (Bode, 2019; Walsh et al., 2020). The simplest HMOs are based on a single lactose molecule, and the galactose residue can be fucosylated (α1–2 linkage) to form 2'-FL or the glucose residue can be fucosylated (α1–3 linkage) to yield 3-FL. Alternatively, the galactose can be sialylated with *N*-acetylneuraminic acid to produce 3'-SL or 6'-SL, depending on the linkage (α2–3 or α2–6, respectively).

The non-reducing end of lactose (galactose) can be elongated and branched with lacto-*N*-biose or *N*-acetyl-lactosamine to produce an array of more complex oligosaccharides that are classified according to the added carbohydrate structure:

- Type I containing lacto-*N*-biose units (Galβ1–3GlcNAc)
- Type II containing *N*-acetyl-lactosamine units (Galβ1–4GlcNAc)

Complex HMOs may feature more than 12 such disaccharide units, each formed by combining any of the five available monomeric units, which can be connected *via* 9 different types of glycosidic bonds (Bode & Jantscher-Krenn, 2012; Kunz, 2012; Kunz et al., 2000). Given this complexity, about 200 different HMOs have been discovered, and the structures of more than 80 are fully solved (Barile & Rastall, 2013). In human milk, 80-85% of the oligosaccharides are neutral, and 15-20% are acidic. Figure 1 summarises the structures of HMOs and Figure 2 shows a typical HMO profile in human milk.

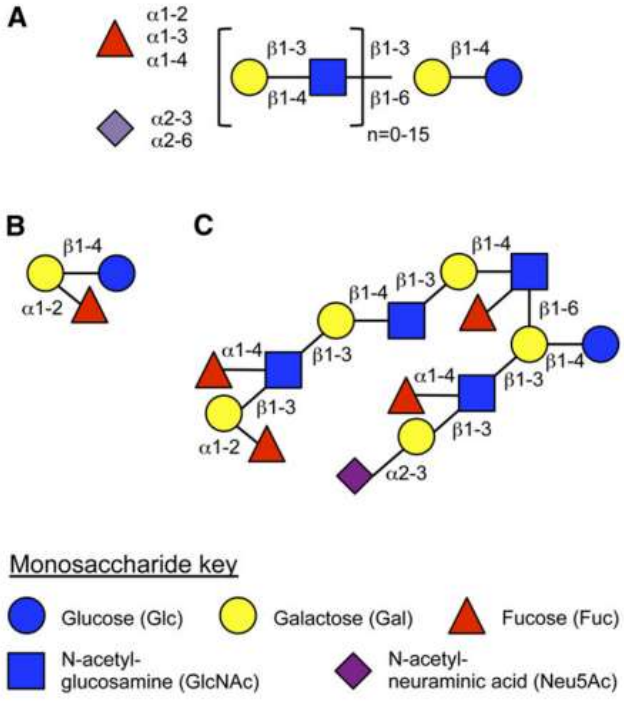


Figure 1: Basic structures of HMOs

Adapted from Bode & Jantscher-Krenn (2012). A) The elements of HMOs: lactose, lacto-*N*-biose (Gal β1–3GlcNAc), *N*-acetyl lactosamine (Gal β1–4GlcNAc), fucose and sialic acid (N-acetyl neuraminic acid, Neu5Ac). B) The core structure lactose is fucosylated or sialylated, to form simple HMOs such as 2'-FL. C) Complex HMOs can be branched and modified like the fucosylated and sialylated ioslacto-*N*-decaose.

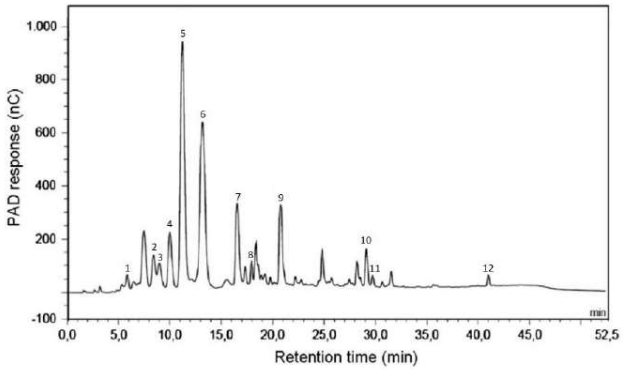


Figure 2: Typical oligosaccharide profile of human milk determined by high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD)

Adapted from Weichert et al. (2013). The chromatogram shows the separation of acidic as well as neutral HMOs. The resulting peaks represent the following oligosaccharides: (1) lacto-*N*-difucohexaose II (LNDFH II), (2) lacto-*N*-fucopentaose III (LNFP III), (3) lacto-*N*-fucopentaose II (LNFP II), (4) lactodifucotetraose (LDFT), (5) lactose, (6) 2´-fucosyllactose (2´-FL), (7) lacto-*N*-fucopentaose I (LNFP I), (8) lacto-*N*-neotetraose (LNnT), (9) lacto-*N*-tetraose (LNT), (10) 6´-sialyllactose (6´-SL), (11) 3´-sialyllactose (3´-SL) and (12) disialyllacto-*N*-tetraose (DS-LNT).

Dietary exposure

2'-FL

Most infants have been exposed to 2'-FL because it is a naturally occurring component of human breast milk. HMO is the third largest component of breast milk solid matter after lactose and lipids and 2'-FL is the most abundant glycan in human breast milk (Castanys-Munoz et al., 2013; Coppa et al., 2004). In a study of milk sampled from women from 10 different countries, 2'-FL was the most abundant sugar (2.38 g/L) and was identified in 85% of the samples. It was found in 100% of the milk sampled from Mexican women, but in only 46% of the milk samples from women from the Philippines and was found at an average concentration of 2.38 g/L (Erney et al., 2000). A subsequent study found that the average concentration of 2'-FL over a lactation period of 50 weeks was 2.43 ± 0.26 g 2'-FL/L of breast milk (Chaturvedi et al., 2001).

Not all infants are exposed to 2'-FL, however, because not all women produce breast milk containing 2'-FL. The fucosylation of glycans depends on the mother's blood group status: Lewis (+)/(-) and Secretor/non-Secretor. The Secretor can synthesize 2'-FL in the mammary gland (Castanyz-Munoz et al., 2013). About 70% of women are Secretors due to the presence of 1-2 fucosyltransferases (FUT2) in their milk (Kunz et al., 1999). The breast milk of non-Secretor women does not contain FUT2 but another fucosyltransferase, α-1,3/4-fucosyltransferase (FUT3), which links Fuc to subterminal GlcNAc in α1-4 linkages (Bode & Jantscher-Krenn, 2012). Secretor Lewis (+) women have the most complex

HMO composition while non-Secretor Lewis (-) women have the least complex (Bode & Jantscher-Krenn, 2012). Because the majority of women are Secretors, infants receiving milk from donor human milk programs are likely to be ingesting 2'-FL. Therefore, infants born to Secretor and non-Secretor mothers are routinely exposed to 2'-FL.

Thurl et al. (2010) found that 2'-FL concentration decreased from day 3 to day 90 of the lactation period from 4.1 to 2.6 g/L, respectively. Though the concentration of 2'-FL declines as lactation continues, the volume of breast milk consumed increases as the infant develops therefore the amount of 2'-FL remains fairly constant throughout the nursing period (Asakuma et al., 2008; Thurl et al., 2010). As a result, an infant up to three months of age born to a Secretor mother may ingest from two to three grams of 2'-FL per day. In addition, the systematic reviews by Thurl et al., (2017), Soyyilmaz et al. (2021) and EFSA (2024) provide a sound compilation of up-to-date data on natural breast milk concentrations for 2'-FL.

Human breast milk is not the only mammalian milk with 2'-FL; domestic goat, sheep, and pig milk contains very small amounts of the neutral oligosaccharide (Albrecht et al., 2014). Though goat milk contains 2'-FL, goat milk has a lower concentration than human milk while cow milk, which does not contain 2'-FL, is commonly used for infant formula (Bode, 2012).

3-FL

The comparative approach is to focus on the concept of substantial equivalence to natural occurring 3-FL, and this data from human breast milk can serve as a blueprint for supplementation levels of the 3-FL with infant formula. The concentration of 3-FL in human breast milk has been quantitated in 27 studies with greater than 5 donors. The results of 10 of these studies were summarised in a systematic review conducted by Thurl et al., (2017). A summary of the findings reported by Thurl et al., (2017) and the 17 additional studies is presented in Appendix 05. In addition, the systematic reviews by Soyyilmaz et al. (2021) and EFSA (2024) provide a sound compilation of up-to-date data on natural breast milk concentrations for 3-FL.

Although 3-FL levels in breast milk vary due to secretor (fucosyltransferase 2, FUT2) status, time postpartum, and geographical location/study population (reviewed in Bode et al., 2012), the available studies show that the concentration of 3-FL in breast milk ranges from 0 - 5.9 g/L, with means and medians ranging from 0 to 2.4 g/L and 0 to 1.1 g/L, respectively. Relying on the systematic review performed by Thurl et al. (2017), the applicant proposes to use the mean value of the individually reported 3-FL levels for 0-6 months of age to set the maximum level for infant formulae (0.9 g/L). Similarly, the 3-FL concentrations reported for >180 days were used for follow-on formulae to better reflect the natural 3-FL levels in breast milk during the later lactation period (1.2 g/kg). The proposed maximum levels are thus within the range of natural levels of human breast milk, which are safely consumed by infants.

LNT

LNT is one of the most abundant oligosaccharides in human milk. Synthetic forms of LNT have also been determined GRAS for use in infant formula and selected conventional foods (GRN 833). Thus, humans are exposed to LNT either through the ingestion of breast milk and/or products containing synthetic forms LNT.

The concentration of LNT in human breast milk has been quantitated in 30 studies with greater than 5 donors. The results of 11 of these studies were summarized in a systematic review conducted by Thurl et al. (2017). A summary of the findings reported by Thurl et al. (2017) and the 17 additional studies is presented in Appendix 05. In addition, the systematic reviews by Soyyilmaz et al. (2021) and EFSA (2024) provide a sound compilation of up-to-date data on natural breast milk concentrations for LNT.

Although the levels of LNT in human milk vary with ethnicity, Secretor and Lewis-blood group status, lactation period, and term vs preterm birth, the available studies show that the concentration of LNT in breast milk generally ranges from 0.003 to 6.7 g/L with means and medians ranging from 0.1 to 3.9 and 0.2 to 2.1 g/L, respectively. Assuming the estimated intake of human milk of 0.8 – 1.2 L per day (EFSA NDA Panel, 2013) by infants of 6,7 kg (EFSA Scientific Committee, 2012), the average daily intake of LNT can be estimated as 0.012 – 0.699 g/kg body weight (bw). Taking into account highest relative consumption on a body weight basis of 260 mL/kg bw/day during the first weeks of life (EFSA Scientific Committee, 2017) a high daily intake of LNT can be estimated as 1.014 g/kg bw. Therefore, the background exposure to LNT from human milk serves as the safe range for the use of Chr. Hansen's LNT in infant formula.

Additionally, a synthetic form of LNT is GRAS at levels up to 0.8 g/L in non-exempt, cow's milk-based infant formula for term infants; 0.6 g/L in drinks for young children (including toddler formulas); 5 g/kg in foods for infants and young children (including toddler foods); 10 g/kg in yogurt; 1 g/L in fluid milk (flavored and unflavored); 2 g/L in meal replacement drinks; 20 g/L in meal replacement bars; 10 g/kg in cereal and granola bars; and 1 g/L in soft drinks, fruit-based -ades, sports drinks, energy drinks, and enhanced waters (GRN 833).

3'-SL

Acidic oligosaccharides make up 15-20% of all HMOs found in human milk (Bode, 2012a). Sialylated HMOs are generated from lactose or other non-sialylated HMOs by sialyltransferases. Sialyllactoses predominantly exist as either 3'-SL or 6'-SL. The concentration of 3'-SL in human breast milk has been analysed by about 22 major studies. Many of these studies were included in the recent systematic review conducted by Thurl et al., (2017). A summary of the results presented by Thurl and colleagues and additional studies not reviewed in Thurl et al., (2017) are presented in Appendix 05. In addition, the systematic reviews by Soyyilmaz et al. (2021) and EFSA (2024) provide a sound compilation of up-to-date data on natural breast milk concentrations for 3'-SL. In the available studies, the average concentration of 3'-SL ranged from 0.08-0.41 g/L. Assuming the estimated intake of 0.8 – 1.2 L per day (EFSA NDA Panel, 2013) by infants of 6,7 kg (EFSA Scientific Committee, 2012), the average daily intake of 3'-SL can be estimated as 0.010 – 0.073 g/kg body weight (bw). Taking into account highest relative consumption on a body weight basis of 260 mL/kg bw/day during the first weeks of life (EFSA Scientific Committee, 2017) a high daily intake of 3'-SL can be estimated as 0.107 g/kg bw. Unlike concentrations of other HMOs, such as 2'-FL and 3-FL, 3'-SL concentrations do not differ between Secretor status of

the mother. Longitudinal studies have demonstrated that 3'-SL concentration stays relatively constant with lactation time (Austin et al., 2016; Kunz et al., 2017; Ma et al., 2018; Sprenger et al., 2017) and although there is some minor variability, 3'-SL levels are relatively consistent across different geographical regions (McGuire et al., 2017).

3'-SL is also found in bovine milk at concentrations ranging from 0.047 – 0.055 g/L and over 1 g/L in bovine colostrum (Aldredge et al., 2013; Urashima et al., 2013; Albrecht et al., 2014). The occurrence level of 3'-SL is approximately six times lower than in human milk (EFSA NDA Panel, 2020a). Unlike in human milk, 3'-SL levels in bovine milk decrease over lactation time and have been found to vary amongst bovine species.

Synthetic forms of 3'-SL have already been approved for use in infant formula in the USA at levels up to 0.238 g/L, 1.6 g/kg for use in baby food products, 25 g/kg in foods for special dietary use, and up to 12.5 g/kg in conventional foods and beverages (GRN 766, 880).

6'-SL

6'-SL is a naturally occurring acidic oligosaccharide found in human milk and is also present at comparable levels in mature bovine, goat, and, to a lesser extent, donkey milk (Martin-Sosa et al., 2003; Claps et al., 2014; Licitra et al., 2019). Thus, humans have been exposed to 6'-SL either through the ingestion of milk from humans or other mammals.

The concentration of 6'-SL in human breast milk has been analysed in 27 studies. The results of 14 of these studies were summarized in a recent systematic review conducted by Thurl et al. (2017). A summary of the findings reported by Thurl et al. (2017) and the thirteen additional studies is presented in Appendix 05. In addition, the systematic reviews by Soyyilmaz et al. (2021) and EFSA (2024) provide a sound compilation of up-to-date data on natural breast milk concentrations for 6'-SL. In the available studies, the average concentration of 6'-SL ranged from 0.1 - 0.8 g/L. Assuming the estimated intake of human milk of 0.8 – 1.2 L per day (EFSA NDA Panel, 2013) by infants of 6,7 kg (EFSA Scientific Committee, 2012), the average daily intake of 6'-SL can be estimated as 0.012 – 0.143 g/kg body weight (bw). Taking into account highest relative consumption on a body weight basis of 260 mL/kg bw/day during the first weeks of life (EFSA Scientific Committee, 2017) a high daily intake of 6'-SL can be estimated as 0.208 g/kg bw. Unlike other HMOs, there is no relationship between Secretor (fucosyltransferase 2, FUT2) status and 6'-SL levels, although there is a large variation in reported values due to differences in time postpartum, geographical location or study population. Most longitudinal studies have demonstrated that 6'-SL concentration decreases with lactation time (2- to 10- fold, depending on the study), although this relationship was not observed in Kunz et al., (2017).

Estimated Daily Intakes

A conservative estimate of the mean and high (95th percentile) consumption levels of infant formula has been derived as **200 mL/kg bw/day** and **260 mL/kg bw/day**, respectively, by the EFSA Scientific Committee (EFSA Scientific Committee et al., 2017). The high consumption value of 260 mL/kg bw/day is considered appropriate for use in the risk assessment of substances which do not accumulate in the

body and are present in foods intended for infants below 16 weeks of age (EFSA Scientific Committee et al., 2017). Similar estimations on the volume of formula consumed daily on a body weight basis have also been employed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for the risk assessment of substances for use in infant formulas (including formulas for special medical purposes intended for infants), such as pectin and octenyl succinic acid (OSA)-modified starch, as examples (Constable et al., 2017; JECFA, 2015, 2017).

The estimated daily intake (EDI) for each of Chr. Hansen's five HMOs from their maximum proposed use levels in infant formula products, as derived based on the estimations of formula consumption volumes, is presented in Table 2 below. The maximum proposed use level values described in this table are not inclusive of manufacturing overages (typically 20%) that are used. Consumption volumes of formula on a body weight basis are highest among infants at around 1 month of age and decline thereafter (Constable et al., 2017; EFSA Scientific Committee et al., 2017). Therefore, the EDI on body weight basis derived using the mean (200 mL/kg bw/day) and high (260 mL/kg bw/day) levels of formula intakes represent the most conservative scenario and are considered appropriate estimates for older infants and toddlers as well.

Even at the high consumption volume of formula of 260 mL/kg bw/day, the estimated daily intake of the five HMOs from its intended uses in formula products (*i.e.*, 780 mg/kg bw/day for 2'-FL, 195 mg/kg bw/day for 3-FL, 390 mg/kg bw/day for LNT, 60 mg/kg bw/day for 3'-SL, and 73 mg/kg bw/day for 6'-SL) are comparable to those of infants fed human milk, as described above in Section 3.1. Although the EDI slightly exceeds the "high" intake levels estimated for 2'-FL (767 mg/kg bw/day) and LNT (287 mg/kg bw/day) by breastfed infants, it should be recognised that:

- Wide variability exists in the concentrations of HMOs in human milk. The upper levels of intake for HMOs by breastfed infants were calculated using "maximum mean" concentrations for the HMOs in mature human milk (reflecting Day 15 to 90 of lactation), as reported in the review by Soyyilmaz et al. (2021). Levels that are even higher than the "maximum mean" concentration for LNT was derived as 1.6 g/L for mature breastmilk, it was reported at 3.9 g/L for transitional milk (reflecting Day 6 to 14 of lactation) (Soyyilmaz et al., 2021). Moreover, in EFSA's safety evaluation of LNT as a novel food, a "high" concentration level of 2.74 g/L was selected for the calculation of the potential exposure to LNT by infants fed human milk (EFSA NDA Panel, 2019b).
- The 95th percentile intake of infant formula consumption used in the derivation of the EDIs (260 mL/kg bw/day) is a highly conservative estimate.

These caveats of the exposure assessment have also been recognised by other authoritative bodies in their safety evaluation of HMOs as formula ingredients. As an example, in a recent opinion on the safety of 3-FL as a novel food published by the EFSA NDA Panel (EFSA NDA Panel, 2021), which included uses in formula products and other foods for infants and young children, it was concluded that:

> *"The Panel also notes that the anticipated daily intake of 3-FL in the NF* [novel food] *from the consumption of IF* [infant formula] *only, in infants up to 16 weeks of age, does not exceed the highest intake level of 3-FL in breastfed infants on a body weight basis. In consideration of the wide variability observed in human milk levels and the conservative assumption underlying the estimated intake, the exceedance at high (95th percentile) intake noted in infants below 1 year*

of age (in only one out of 13 dietary surveys included in the EFSA food consumption database) does not raise safety concerns."

Table 2: Estimated daily intake for HMOs in infant formula products

HMO	Maximum Proposed Use Level (g/L)[1]		Estimated Daily Intake of HMOs (mg/kg bw/day)[2]		Estimated Daily Intake of HMOs (g/day)			
	Infant Formula	Follow-on Formula & SMPPi			Infants (0-6 months)[3]		Infants (6-12 months)[3]	
			Mean	95th	Mean	95th	Mean	95th
2'-FL	3.0	3.64	600	780	3.6	4.7	5.4	7.0
3-FL	0.9	1.2	150	195	0.9	1.2	1.4	1.8
LNT	1.82	1.82	300	390	1.8	2.3	2.7	3.5
3'-SL	0.28	0.28	46	60	0.3	0.4	0.4	0.5
6'-SL	0.7	0.7	56	73	0.3	0.4	0.5	0.7

Abbreviation(s): bw, body weight; EDI, estimated daily intake; HMOs, human milk oligosaccharides.

[1] The maximum proposed use level values described in this table are not inclusive of manufacturing overages (typically 20%) that are used.

[2] Calculated as: Use Level (mg/mL) * Formula Consumption (mL/kg bw/day). Formula consumption levels of 200 mL/kg bw/day (mean) and 260 mL/kg bw/day (95th percentile) are considered appropriate for use in the risk assessment of substances present in foods intended for infants below 16 weeks of age (EFSA Scientific Committee et al., 2017).

[3] Calculated as: EDI (mg/kg bw/day) * default body weight / 1000. For infants, a default body weight was assumed to be 6 kg for infants 0 to 6 months, and 9 kg for infants 7 to 12 months (Institute of Medicine, 2005).

2'-FL and 3-FL

Fucosylated oligosaccharides are also common structures found on glycolipids and proteins with N-linked or O-linked glycans, and the fucosyl moiety is often the terminal modification of the glycan structure. These glycan structures are involved in many biological processes such as cell adhesion, cell differentiation and cell growth, host-microbe interactions, immune reactions, and cell signalling. Therefore, humans are exposed to fucosylated oligosaccharides through both ingestion in early life and through endogenous production.

HMOs modified with fucose or sialic acid share structural motifs with the histo-blood group antigens (HBGA) found on the surface of the intestinal epithelium. These glycans act as epithelial receptors to which pathogens can adhere. The surface of bacteria exhibits an array of complex glycan structures (adhesins) that specifically bind to these receptors on the host mucosa.

Fucosylated HMOs such as 2'-FL mimic glycans on epithelial receptors such as certain HBGAs. The synthesis of glycans like HBGAs and HMOs follows a similar blueprint and is catalysed in part by the same enzymes (Becker & Lowe, 2003; Blank et al., 2012; Bode and Jantscher-Krenn, 2012; Marionneau et al., 2001). HBGA structures are present at the surface of erythrocytes and in secretions such as milk and saliva, but also in mucins, a complex class of glycoproteins that is secreted by goblet cells in the human epithelium.

Fucosyltransferases catalyse the formation of glycosidic bonds between nucleotide-activated fucose (GDP-fucose) and acceptor molecules, such as galactose and *N*-acetylglucosamine that decorate glycoproteins and glycolipids. Thirteen fucosyltransferases have been identified in humans (Becker & Lowe, 2003). *FUT1* and *FUT2* encode α(1,2)-fucosyltransferases (Fut1 and Fut2) that are responsible for the synthesis of the H-antigen and related structures. The H-transferase (*FUT1* gene product) is expressed in erythroid precursors. In individuals of blood group A, B or AB, the H-antigen must be further modified, whereas unmodified H antigen is expressed at cell surfaces of type 0 individuals. The *FUT2* gene, also called secretor gene (*Se*), determines secretor status. The *FUT2* protein, Fut2, catalyses the addition of fucose in 1→2-glycosidic bond to type 1 precursor (Gal-β(1,3)-GlcNAc), or other precursors such as lactose. *FUT2* is expressed in glandular epithelial tissues such as the mammary and salivary glands, including the endothelium. The resulting H type I trisaccharide Fuc-α(1,2)Gal-β(1,3)GlcNAc is the precursor for the ABO and Lewis b type HBGAs, and is a core structure for more complex HMOs (Huang *et al*., 2003). The biosynthetic pathway of HBGA structures is depicted in Figure 3. *FUT3-7* and *FUT9* encode fucosyltransferases that synthesise α(1,3) glycans. FUT3, also known as Lewis-fucosyltransferase, synthesises α(1,3) and (1,4)-glycans, such as the LEWISx and sialyl LEWISx antigens (Becker & Lowe, 2003).

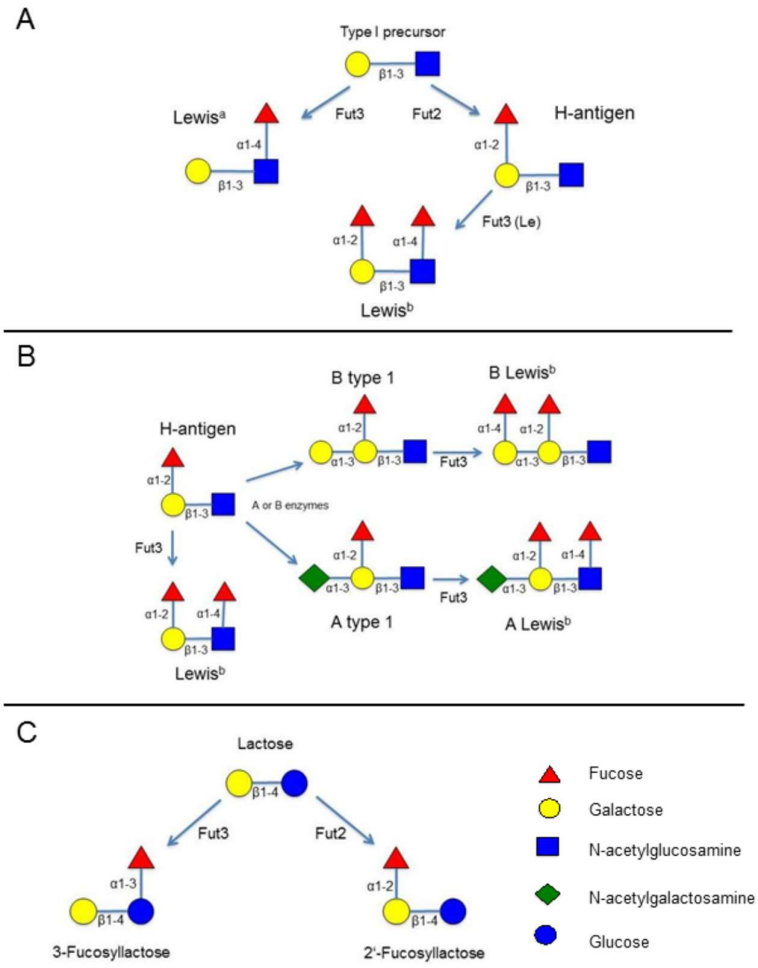


Figure 3: Major biosynthetic pathway of fucosylated ABH and Lewis antigens based on the type I precursor disaccharide (adapted from Le Pende, 2004)

A: Secretor (Fut2) and Lewis-type (Fut3) human blood group antigen (HBGA) based on precursor type I. B: ABO-type HGBA based on H-type. C: Synthesis of 2'-fucosyllactose and 3-fucosyllactose from lactose.

Beneficial properties of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL

Several studies have shown that the consumption of fucosylated HMOs from breastmilk are associated with changes in the microbiota composition in infants (Korpela et al., 2018). Additionally, a study by Holst et al., (2023) showed that consumption of infant formula with 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL shifted the microbiome composition of formula-fed infants to that of breastfed infants. This shift was characterised by the increased relative abundance of bifidobacterial and a lower abundance of potentially pathogenic bacteria (Holst et al., 2023).

Additionally, studies have also suggested that HMOs are able to support intestinal barrier function by several mechanisms, including modified epithelial cell gene expression, proliferation, and differentiation (Donovan & Comstock, 2016).

Other beneficial properties of HMOs include anti-infective activity, modulatory effects on the immune system, and effects on neurodevelopment.

A more detailed description of the beneficial properties of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL is described in Appendix 06.

History of safe consumption from human milk

Human breastmilk is widely recognized as the optimal form of nutrition for infants, with exclusive breastfeeding recommended for the first six months of age and continued breastfeeding with complementary foods to two years of age and beyond (Eidelman & Schanler, 2012; Kramer & Kakuma, 2012; Pound et al., 2012; WHO, 2021). Human milk contains a highly abundant and unique fraction of structurally diverse glycans known as HMOs (Bode, 2012). HMOs are present at high concentrations in breastmilk, representing the third most abundant solid component after lactose and total lipids (Bode, 2012, 2019). Total concentrations of HMOs are reported in the ranges of 20 to 25 g/L in colostrum, and up to 20 g/L in mature human milk (Bode, 2012). Maternal genetic factors (*i.e.*, allelic variations in the Secretor and Lewis genes) are a key determinant of the HMO composition of human milk, though various other factors (such as lactation stage) may also play a role (Bode, 2019; Han et al., 2021; Walsh et al., 2020).

While HMOs represent a large component of human breastmilk, they occur only at low concentrations in cow milk, which is commonly used to formulate infant formula (Albrecht et al., 2014). Accordingly, manufactured versions of purified HMOs have been developed as ingredients in infant formula. The maximum proposed use levels of Chr. Hansen's HMOs in formula products were selected on the basis that they are comparable to concentrations of their naturally occurring counterparts in human milk. Detailed analyses of the concentrations of different HMOs present in human milk have been evaluated in a systematic review conducted by Thurl et al. (2017). Although this was intended as a comprehensive review, there were some limitations in the methodologies applied by the study authors. For instance, the review excluded publications that: reported only median concentration levels of HMOs or analysed milk samples at "*lactation periods not fitting the lactation periods defined in this review*".

More recently, another comprehensive review derived representative HMO concentrations that are reflective of those in pooled milk samples from healthy mothers, in order to provide a more global estimate of HMO levels in human milk throughout lactation, regardless of individual variations resulting from genetic and non-genetic factors (Soyyilmaz et al., 2021). In this review, 57 peer-reviewed primary publications published between 1996 and 2020 that reported the concentrations of individual HMOs in human milk were included for assessment. Since the main focus of the review was to capture the representative mean levels of HMOs within pooled milk samples, for the primary publications where HMO concentrations were reported separately by milk groups or secretor status, conversion factors were applied by the review authors to derive the levels that would be expected in a pooled sample (e.g., assuming 80%/20% frequency of secretors/non-secretors in the population). Descriptive statistics were used to summarize the different mean HMO concentrations obtained in each of the studies evaluated in the review (e.g., "mean of means", "maximum mean"). Using a different approach, another recent review article derived weighted means, standard deviations, medians, interquartile ranges, 90th percentiles, and probability distribution using random sampling of

the statistical data reported for HMO concentrations in human milk reported in primary research papers (Conze et al., 2022). The weighted means and 90th percentile values derived for each of the HMOs in this review are comparable to those that were reported by Soyyilmaz et al. (2021). Across both reviews, the primary research articles evaluated HMO concentrations in the milk of mothers located across a wide range of regions (*e.g.*, North America, Latin America, Europe, Asia-Pacific, Africa) (Conze et al., 2022; Soyyilmaz et al., 2021). In addition, the systematic review by EFSA (2024) provides a sound compilation of up-to-date data on natural breast milk concentrations for HMOs.

Based on the concentrations of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL that have been reported in human milk (see Table 3), the estimated daily intake of these five HMOs by infants exclusively fed human milk can be derived (see Table 4). For this calculation, the HMO concentrations in the mature milk of mothers (lactation day 15 to 90) reported by Soyyilmaz et al. (2021) were selected since it reflects the period during which infants would most likely be consuming human milk, or otherwise infant formula, as their sole source of nutrition. As illustrated in Table 4, the maximum proposed use levels of Chr. Hansen's HMOs in formula are within the concentration ranges of these HMOs in human milk, including the "maximum mean" values reported across different studies. Accordingly, the maximum proposed use levels of Chr. Hansen's HMOs in formula will result in similar levels of intakes as those ingested by breastfed infants consuming these same HMOs through human milk, which helps to support their safety.

Table 3: Concentrations of HMOs in human milk reported in published reviews

HMO	Concentrations in Human Milk Selected for the Safety Evaluations of HMOs Conducted by the EFSA NDA Panel[1]			Concentrations in Mature Milk (15-90 days) Reported in the Soyyilmaz et al., 2021 Review		Concentrations in Human Milk Based on the Conze et al., 2022 Review	Concentration in Human Milk Based on the Malih et al., 2024 Review			Maximum proposed use level of Chr. Hansen's HMOs (g/L)	
	Reference	Mean (g/L)	High (g/L)	Mean of means (g/L)	Maximum Mean (g/L)	Mean (g/L)	90th Percentile (g/L)	Mean of means (g/L)	Maximum mean (g/L)	Infant Formula	Follow-on Formula & SMPPi
2'-FL	Erney et al., 2001	2.38	4.78	2.28	4.28	2.58	4.32	1.69	8.40	3.0	3.64
3-FL	Thurl et al., 2017	1.24	1.44	0.72	1.90	0.57	1.4	0.91	5.00	0.9	1.2
LNT	Erney et al., 2001	0.76	2.74	0.74	1.60	0.94	1.76	1.06	3.90	1.82	1.82
3'-SL	Thurl et al., 2017	0.29	0.36	0.19	0.70	0.28	0.49	0.23	1.22	0.28	0.28
6'-SL	Thurl et al., 2017	0.66	1.08	0.40	0.74	0.39	0.77	0.44	3.34	0.7	0.7

[1] The mean and "high" concentration values listed in this table were selected by the EFSA NDA Panel during their safety evaluation of 2'-FL (EFSA NDA Panel, 2019a), 3-FL (EFSA NDA Panel, 2021), LNT (EFSA NDA Panel, 2019b), 3'-SL (EFSA NDA Panel, 2020a), and 6'-SL (EFSA NDA Panel, 2020b).

Table 4: Estimated daily intake of HMOs by infants exclusively fed human milk

HMO	Concentration in Human Milk (g/L) Reported by Soyyilmaz et al., 2021	Estimated Daily Intake of HMOs from Human Milk (mg/kg bw/day)[1,2]	
		Milk Volume: 800 mL/day	Milk Volume: 1,200 mL/day
2'-FL	Mean of Means: 2.28	272	408
	Maximum Mean: 4.28	511	767
3-FL	Mean of Means: 0.72	86	129
	Maximum Mean: 1.90	227	340
LNT	Mean of Means: 0.74	88	133
	Maximum Mean: 1.60	191	287
3'-SL	Mean of Means: 0.19	23	34
	Maximum Mean: 0.70	84	125
6'-SL	Mean of Means: 0.40	48	72
	Maximum Mean: 0.74	88	133

Abbreviation(s): 95% CL, 95% confidence limit; bw, body weight.

[1] The average volume of human milk consumed by infants has been estimated at 800 mL/day, with an upper bound of 1,200 mL/day (EFSA NDA Panel, 2013; Institute of Medicine, 1991). Default body weight for infants was assumed to be 6.7 kg (EFSA Scientific Committee, 2012).

[2] Calculated as: (Concentration of HMO in Human Milk) * (Milk Volume Ingested) / (Default Body Weight)

A.2 General data requirements for supporting evidence

Chr. Hansen confirms that the 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL substances are representative of the commercial products on which approval is sought. The scientific evidence for potential beneficial physiological or health-related outcomes presented in the application is based on human studies containing 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL, with the focus on infants being the target population group.

B. Technical information on the use of the nutritive substance

B.1 Information to enable identification of the nutritive substance

Chr. Hansen intends to market 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL, for use alone and/or in combination in infant formula products. Each of the five HMOs are first independently produced *via* separate microbial fermentation processes and are ready to be used or subjected to a mixing step to make a 5HMO-Mix blend. The structures of each of these HMOs is presented below in Figure 4 and Table 5.

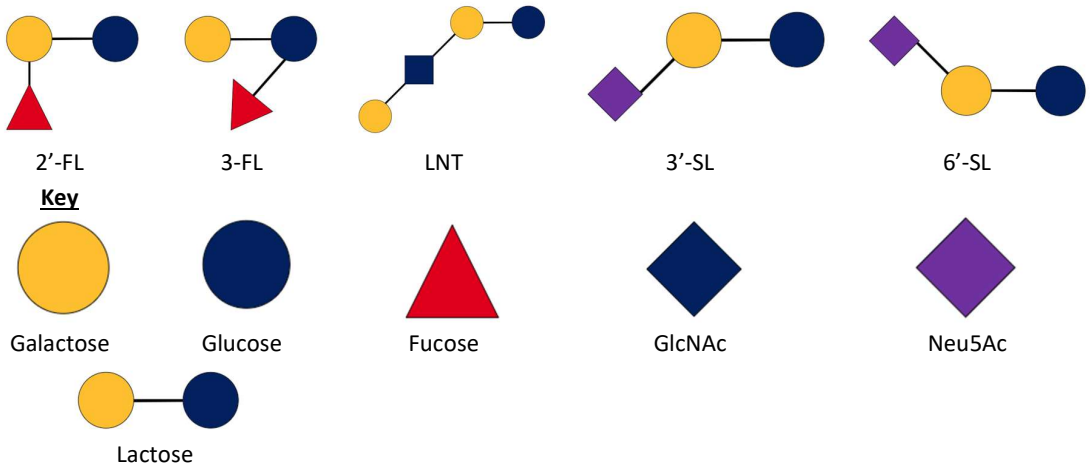
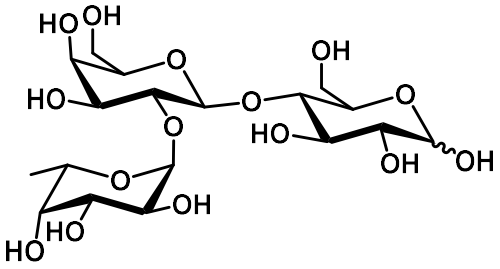
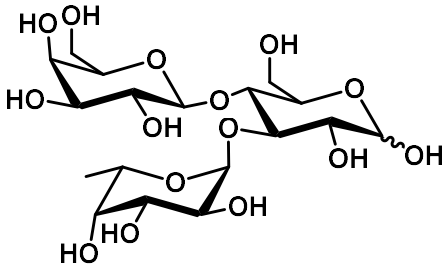
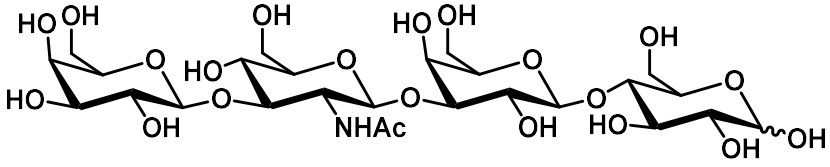
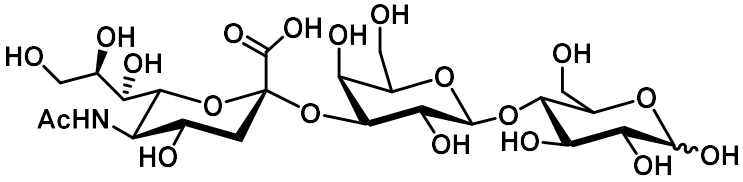
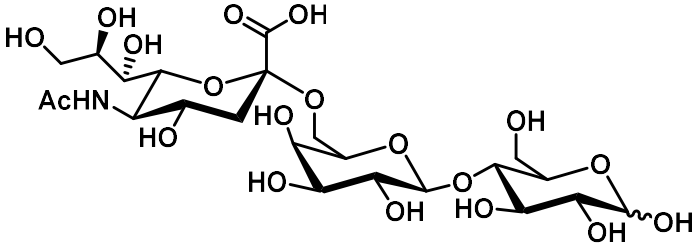


Figure 4: Principal linkages of the five HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL)

Table 5: Chemical structures of 2'-L, 3-FL, LNT, 3'-SL, and 6'-SL

2’-Fucosyllactose (2’-FL) 	3-Fucosyllactose (3-FL) 
Lacto-*N*-tetraose (LNT) 	
3’-Sialyllactose (3’-SL) 	
6’-Sialyllactose (6’-SL) 	

B.2 Information on the chemical and physical properties of the nutritive substances

B.2.1 Chemical and physical properties of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL

Table 6: CAS number, chemical names, and chemical properties of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL

HMO	CAS Number	Chemical Name and Corresponding Chemical Abbreviations	Empirical Formula	Molecular Mass[1] [g/mol or Da]
2'-Fucosyllactose	41263-94-9	• α-L-Fucosypyranosyl-(1→2)-β-D-galactopyranosolyl-(1→4)-D-glucopyranoside • α-L-Fuc-(1→2)-β-D-Gal-(1→4)-D-Glc • Fuc-α-1,2-Gal-β-1,4-Glc	$C_{18}H_{32}O_{15}$	488.439
3-Fucosyllactose	41312-47-4	• β-D-Galactopyranosyl-(1→4)- [α-L-fucopyranosyl-(1→3)]- D-glucopyranose • β-D-Gal-(1→4)-(α-L-Fuc-(1→3))-D-Glc • Galβ1-4(Fucα1-3)Glc	$C_{18}H_{32}O_{15}$	488.439
Lacto-*N*-tetraose	14116-68-8	• β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose • β-D-Gal-(1→3)-β-D-GlcNAc-(1→3)-β-D-Gal-(1→4)-D-Glc • Galβ1-3GlcNAcβ1-3Galβ1-4Glc	$C_{26}H_{45}NO_{21}$	707.632
3'-Sialyllactose (sodium salt)	128596-80-5	• N-Acetyl-α-D-neuraminyl-(2→3)-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt • α-Neu5Ac-(2→3)-β-D-Gal-(1→4)-D-Glc sodium salt • Neu5Acα2-3Galβ1-4Glc sodium salt	$C_{23}H_{38}NO_{19}Na$	655.53
6'-Sialyllactose (sodium salt)	157574-76-0	• N-Acetyl-α-D-neuraminyl-(2→6)-β-D-galactopyranosyl-(1→4)-D-glucose, sodium salt • α-Neu5Ac-(2→6)-β-D-Gal-(1→4)-D-Glc sodium salt • Neu5Acα2-6Galβ1-4Glc sodium salt	$C_{23}H_{38}NO_{19}Na$	655.53

[1] Taken from PubChem

Table 7: Physical properties of the 5HMO-Mix

Parameters	5HMO-Mix
Appearance and colour	Powder white to ivory
Powder properties	Fine hydroscopic powder
Taste	Sweet (caramel-like)
Smell	Neutral (milk-like)
Solubility	Min. 500 g/L in water at ambient temperature (for 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL individually or as combined in the 5HMO-Mix)

B.2.2 Structural equivalence to naturally occurring HMOs

Proton and carbon NMR spectroscopy analyses demonstrate Chr. Hansen's five HMOs ingredients are chemically equivalent to their corresponding HMO counterparts isolated from human milk. Additionally, complete ^{13}C and ^{1}H NMR spectra assignment was made for all five HMO ingredients. Details of these analyses are provided in Appendix 07 (**COMMERCIAL CONFIDENTIAL INFORMATION**).

Identity analysis of Chr. Hansen's HMOs was further performed based on collision induced decay (CID) and fragmentation pattern of oligosaccharides by comparison with highly purified standards. Oligosaccharides were profiled using a high-performance liquid chromatography coupled mass spectrometry (LC/MS) workflow incorporating targeted acidic and neutral carbohydrate analysis. Thereby oligosaccharides were fractionated by LC with respect to their polarity following a targeted online MS. Also, oligosaccharides were determined based on their diagnostic signature fragments by multiple reaction monitoring (MRM). The identity of Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL was confirmed, and structural equivalence to commercially available standards was demonstrated. Details of the mass spectra analyses are also presented in Appendix 07 (**COMMERCIAL CONFIDENTIAL INFORMATION**).

B.2.3 Stability

Genetic stability of the production strain

To ensure genomic stability and finished product batch-to-batch consistency, all modifications that were introduced into the *E. coli* BL21(DE3) production strains were stably integrated, and the production of Chr. Hansen's HMOs occurs in a sterile environment. Thus, the production strains are not expected to lose their ability to produce a consistent finished product. Further details are provided in Appendix 08 (**COMMERCIAL CONFIDENTIAL INFORMATION**).

Stability of individual HMOs

Real-time stability tests were carried out on representative batches of individual samples of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL. The samples were taken at different time points for the determination of the respective HMO and moisture content (base value of 100 was taken as a value measured at month 0).

The test results as provided in Appendix 09 (**COMMERCIAL CONFIDENTIAL INFORMATION**) show that the products remain stable and safe for use up to 27 months after production.

Stability of the 5HMO-Mix

A study is currently being conducted to evaluate the stability of the powdered 5HMO-Mix and its single HMOs. The interim study report, which includes data obtained following 156 weeks (3 years) of storage at the ambient conditions (25°C and 60% relative humidity), and 26 weeks (6 months) of storage at accelerated conditions (40°C and 75% relative humidity), is provided in Appendix 10 (**COMMERCIALLY CONFIDENTIAL INFORMATION**). One representative batch of the 5HMO-Mix was aliquoted, and samples were analysed at designated time intervals for the content of each of the five individual HMOs (by HPAEC-PAD) and moisture (Karl-Fischer titration).

On a dry weight basis, the total and relative concentrations of the five HMOs in the 5HMO-Mix remained relatively unchanged over time. It was concluded that 5HMO-Mix is stable for at least 2 years when stored at 25°C and 60% relative humidity. Details of this study are available in Appendix 10 (**COMMERCIAL CONFIDENTIAL INFORMATION**).

The stability of individual HMOs in infant milk powder containing 5 HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) was investigated by a downstream customer. The results of this study showed that the HMOs remained stable in the infant milk powder at room temperature for two years. The study is briefly described in Appendix 09 (**COMMERCIAL CONFIDENTIAL INFORMATION**).

A separate stability study was conducted in infant formula containing the 5HMO-Mix. The study was conducted using a commercial infant formula for 6 months at room temperature. The results of this study indicated that neither the compositional analysis nor the investigation of the pH value indicated instability. The technical report is attached as Appendix 18 (**COMMERCIAL CONFIDENTIAL INFORMATION**).

B.3 Information on the impurity profile

B.3.1 Chemical considerations

Chr. Hansen's 5HMO-Mix blend and its single HMOs meets defined specifications, which sets forth acceptable limits for residual carbohydrate by-products, proteins, and recombinant DNA, as well as potential contaminants such as heavy metals, endotoxins, and aflatoxins (see **Section 3.3.3 B.5**). Analytical data from representative production batches of 5HMO-Mix demonstrate conformity to these specifications (see **Section B.5**). HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) produced with genetically modified strains of *E. coli* BL21(DE3), and demonstrated they are chemically equivalent to their naturally occurring counterparts in human milk (see **Section 3.3.3 B.2.2** and Appendix 07 (**COMMERCIAL CONFIDENTIAL INFORMATION**)).

Analytical data from representative production batches of 5HMO-Mix demonstrate conformity to these specifications (see **Section 3.3.3 B.5**).

B.3.2 Microbiological considerations

Chr. Hansen's HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) are all produced with derivatives of *E. coli* BL21(DE3), which is considered a safe and non-pathogenic organism. *E. coli* BL21(DE3) does not possess the fertility plasmid indicating that it is incapable of transferring its DNA to other organisms, and it is also incapable of colonizing the human gastrointestinal system.

At the end of each fermentation run, the fermentation medium containing the individual HMO of interest (*i.e.*, 2'-FL, 3-FL, LNT, 3'-SL, or 6'-SL) is separated from the bacterial biomass by e.g., centrifugation or filtration. Live bacteria in the biomass are inactivated either chemically or thermally according to applicable EU law prior to disposal. In addition to establishing the criteria for microbiological contaminants, the specifications for Chr. Hansen's 5HMO-Mix, and its single HMOs include acceptable limits for endotoxins and residual protein (see **Section 3.3.3 B.5**). Furthermore, no residual DNA from the production strains are present in the 5HMO-Mix, as confirmed using a sensitive qPCR assay (see **Section 3.3.3 B.5** and Appendix 11 (**COMMERCIAL CONFIDENTIAL INFORMATION**)). The final production strains and optional degradation strains listed below do not contain plasmids or other episomal vectors and are not capable of DNA transfer to other organisms.

- For 2'-FL:
  ***Escherichia coli* BL21(DE3) containing the gene for alpha-1,2-fucosyltransferase from *Escherichia coli* O126**

- For 3-FL:
  Escherichia coli* BL21(DE3) containing the gene for alpha-1,3-fucosyltransferase from *Bacteroides fragilis

- For LNT:
  Escherichia coli* BL21(DE3) containing the genes for *N*-acetylglucosaminyltransferase from *Neisseria meningitidis* and for beta-1,3-galactosyltransferase from *Salmonella enterica

  and optionally used degradation strain:
  Escherichia coli* BL21(DE3) containing the gene for beta-*N*-acetylhexosaminidase from *Bifidobacterium bifidum

- For 3'-SL:
  Escherichia coli* BL21(DE3) containing the gene for alpha-2,3-sialylltransferase from *Haemophilus parahaemolyticus

- For 6'-SL:
  Escherichia coli* BL21(DE3) containing the gene for alpha-2,6-sialylltransferase from *Streptococcus suis

- For 3'-SL and 6'-SL optionally used degradation strain:
  ***Escherichia coli* BL21(DE3) containing the gene for *N*-acetylglucosamine-6-phosphate deacetylase from *Escherichia coli* BL21(DE3)**

B.3.3 Absence of amino acids and biogenic amines

The parental strain to the production organism is *E. coli* BL21 which is a strain with no known toxicity. A study conducted by Chart et al. in 2000 revealed that *E. coli* BL21 strains do not carry the pathogenic mechanisms required to cause enteric infections (Chart et al., 2000). According to the German Federal Office of Consumer Protection and Food Safety the parental strain *E. coli* BL21 is a biosafety level 1 strain (*Federal Office of Consumer Protection and Food Safety*). These data indicate that *E. coli* BL21 does not produce toxicologically relevant secondary metabolites.

Furthermore, the parental strain *E. coli* BL21 is not known to be prone to produce biogenic amines. It is possible that the production of biogenic amines could be enabled through the introduction of new genes during the genetic modification of the strain. However, even in case that any potential biogenic amines would be present, they would be depleted during the downstream process of the production. The depletion takes place at the ion exchangers; due to the low pH during the process, the amines would be protonated and exchanged as cations against H^+ in the cation exchange column.

Similarly, the majority of potentially present amino acids would be removed from the product through the ion exchangers, as well. Additionally, the proposed specification for proteins in 5HMO-Mix is very low (≤ 100 µg/g) and especially basic amino acids are detected with the protein detection method used, a modified Bradford assay.

Even in the case that small amounts of secondary metabolites such as biogenic amines and amino acid would be present in the novel food after the downstream processing, the toxicological and clinical studies shared in **Section 3.3.3 C** of this application shows that the 5HMO-Mix is not mutagenic or genotoxic, did not lead to any adverse events in a 90 day sub-chronic rodent toxicity study and that the consumption of a 5-HMO mix containing infant formula (5.75 g 5HMO-Mix/L) is safe and well-tolerated for infants (Parschat et al., 2020; 2021).

In summary, the parental strain is known to be safe and potentially present biogenic amines and amino acids and similar molecules would be removed during the ion chromatography steps during downstream processing. The absence on such secondary metabolites is further supported by the low protein content of the novel food. Additionally, toxicological studies as well as a clinical study with infants on the novel food showed no adverse effect, which makes us confident to state that there are no amounts of secondary metabolites present in the final product which would raise any safety concern.

B.3.4 Absence of the production organism and its DNA

With regards to the production strains employed, the bacteria are removed during downstream purification processes, and no residual DNA or viable cells from the production strains remain in the finished HMO preparations. Both was proven by a sensitive qPCR assay which shows the absence of DNA, and a test which shows the absence of viable cells of the production strains in HMOs products.

With regards to residual DNA, the absence of recombinant genetic material in the 5HMO-Mix was confirmed by quantitative PCR. Every single batch of the 5HMO-Mix undergoes this analytical test

routinely, and the sample applies for single HMO batches. As the qPCR target genes are distributed throughout the chromosomes of the production strains, their absence in the final product is a universal and sensible marker for the absence of any residual DNA from the genetically modified *E. coli* B21(DE3) production strains. A real-time quantitative PCR (qPCR) method was developed and validated to detect strain specific genes. The validation reports and exemplary test report for these analyses in 5HMO-Mix are provided in Appendix 12 (**COMMERCIAL CONFIDENTIAL INFORMATION)**.

The testing to demonstrate the absence of viable cells of the *E. coli* BL21(DE3) production strains in HMO products was conducted according to ISO 4832:2006-02 (Appendix 13 (**COMMERCIAL CONFIDENTIAL INFORMATION)**). The aim was to check for the presence of *E. coli* cells. The test involved simultaneous examination in selective and non-selective media, with the addition of the HMO production strains to sample dilutions. Samples were taken from three batches per HMO and incubated for up to 64 hours. Results showed colonies on petri dishes of the positive controls, indicating no inhibitory effect of the culture medium or product substrate. Most samples showed no colony growth. MALDI-TOF MS was used for identification, revealing organisms of no concern for food safety in the colonies. This bacterium identified is common in dry food products, poses no safety concern, and is negligible in the HMO product as long as specifications are met. Although microbial colonies were found, they were not identified as *E. coli*. Therefore, no viable cells of the production strains were detected in the tested batches.

In conclusion the present results of the qPCR, and absence of viable cells of the production strains analyses are supporting the evidence that HMOs products does not contain transgenic gene fragments and genetically modified microorganisms.

B.3.5 Residual anions and trace elements

The safety of the substance used for a nutritive purpose concerning contaminants like heavy metals or minerals is assured through the downstream processing in production process. The ion exchange chromatography and electrodialysis steps remove ions remaining from the fermentation media. Moreover, the safety of 5HMO-Mix has been shown trough toxicological and clinical studies as described in **Section 3.3.3 C**.

Further routine analyses of relevant heavy metals are performed and showing the absence of heavy metals in the final product (see **Section 3.3.3 B.3.6**).

Therefore, it's concluded that no residual anions and trace present are present in the 5HMO-Mix.

B.3.6 Heavy metals

Chr. Hansen analyses the minerals arsenic, cadmium, lead, and mercury as these are the only minerals that are regulated with upper limits in the regulation on contaminants (EU) 2023/915, although these limits are technically not applicable for HMOs, but for the final product like infant formula. In the EU, Chr. Hansen's HMOs only includes these heavy metals in its specification.

The specifications for FSANZ-approved HMOs produced by other companies and/ or other production organisms is detailed in S3—2 of Schedule 3 – Identity and Purity. Furthermore, S3—4 provides

additional and supplementary requirements with regards to heavy metal limits for lead (2 mg/kg), arsenic (1 mg/kg), cadmium (1 mg/kg), and mercury (1 mg/kg).

The specifications for Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are presented in Table 9 below. These results indicate that the measurements for lead, arsenic, cadmium, and mercury are well below the specifications provided by FSANZ in Schedule 3 – Identity and Purity.

The safety of the substance used for a nutritive purpose concerning contaminants like heavy metals or minerals is assured through the downstream processing in production process. The ion exchange chromatography and electrodialysis steps remove ions remaining from the fermentation media. Moreover, the safety of 5HMO-Mix has been shown trough toxicological and clinical studies as described in **Section 3.3.3 C**.

B.4 Manufacturing process

Manufacturing process for individual HMOs

The production processes of individual 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are compliant to European regulation (EC) No. 178/2002 laying down the general principles and requirements of food law. Consequently, production in the EU is controlled by the Food Hygiene Regulation (EC) No. 852/2004. Individual 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are manufactured in accordance with current Good Manufacturing Practice for Food (GMP) and the principles of Hazard Analysis of Critical Control Points (HACCP).

In Appendix 14 (**COMMERCIALLY CONFIDENTIAL INFORMATION**), the certificates proving the compliance of the production site with FSSC 22000 standard can be found. Compliance with Food Hygiene Regulation is regularly controlled by relevant food inspection services.

The manufacturing process can be divided into three main steps as described below:

1. Step 1: Upstream processing (i.e., fermentation process of the individual HMOs)
2. Step 2: Downstream processing (i.e., the recovery and purification of the individual HMOs)
3. Step 3: Drying (e.g., spray-drying of the final product powder)

All raw materials and processing aids used in the upstream and downstream processes for 3-FL, LNT, 3'-SL, and 6-SL are provided in Appendix 08 (**COMMERCIAL CONFIDENTIAL INFORMATION**) and are approved for the manufacture of food. The manufacturing process for 2'-FL has previously been submitted in Application A1190.

All equipment with product contact used to produce the individual HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) are compliant to European Regulation (EC) No. 1935/2004 on materials and articles intended to come into contact with food like stainless steel or plastic materials according to European Regulation (EU) No. 10/2011 on plastic materials and articles intended to come into contact with food. In process controls (IPCs) as well as analyses of the intermediate and final product are performed according to validated and accredited methods (e.g., analysing for carbohydrate profile, HMO purity in dry matter, moisture, protein content, contaminants, heavy metals and toxins, and microbiology).

Additional details on the manufacturing process for 3-FL, LNT, 3'-SL, and 6'-SL are provided in Appendix 08 (**COMMERCIAL CONFIDENTIAL INFORMATION**). The manufacturing process for 2'-FL has previously been submitted to FSANZ in Application A1190.

Manufacturing process for 5HMO-Mix

Production of 5HMO-Mix occurs in a contained environment. The manufacturing process is conducted in accordance with Good Manufacturing Practice (GMP). Chr. Hansen's production facilities are FSSC 22000-certified and fully implemented Hazard Analysis Critical Control Point (HACCP) plans, standard operating procedures, and quality control programs are in place to ensure quality of the finished product.

Each of the five HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) are independently produced via separate microbial fermentation with genetically modified strains of *E. coli* BL21(DE3). The manufacturing process for the five individual HMOs are basically identical except for a few process parameters. Fermentation is initiated in minimal medium with a defined carbon source in a seed fermenter, and is continued in a main fermenter, where the lactose substrate is added either only in the batch phase of the fermentation or throughout the fermentation process. The process is carried out without inhibitors, inducers or antibiotics and no solvents are used except water. The duration of fermentation time is set to optimize the HMO concentrations and is prudently monitored. The production strains export the HMOs into the fermentation medium, and the bacterial biomass is then separated from the culture broth, which contains the HMOs. The isolation, purification and concentration of the HMOs involve several filtration steps, ion exchange, electrodialysis and activated carbon treatment.

The concentrated HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) are dried to obtain a highly purified powder that is ready for commercialisation. As indicated in Figure 5, the dried HMO powders can be blended by dry mixing to produce 5HMO-Mix. Alternatively, the individual HMOs in solution can be wet-blended and the mixed solution is spray dried to yield the powdered 5HMO-Mix ingredient. Another option is to re-dissolve the dried individual HMOs in water and wet-blend in solution before a final drying process.

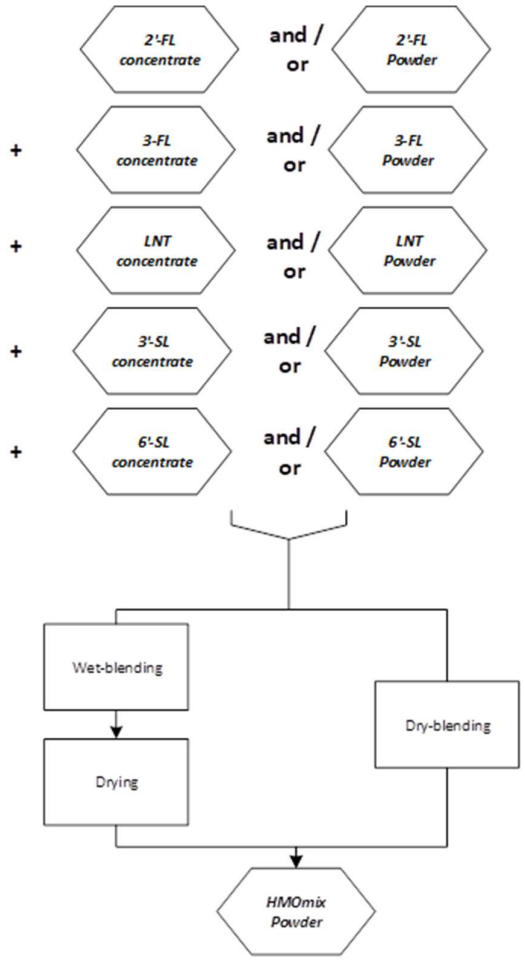


Figure 5: Overview of the manufacturing process for Chr. Hansen's 5HMO-Mix

B.5 Specification for identity and purity

Chr. Hansen has established specifications for the 5HMO-Mix, as outlined in Table 8. The 5HMO-Mix is specified to contain a defined amount of 2'-FL (52 ± 5 %), 3-FL (13 ± 3 %), LNT (26 ± 3 %), 3'-SL (4 ± 1 %), and 6'-SL (5 ± 1 %). The remaining components comprise small amounts of inorganic material (ash), moisture, and residual carbohydrate by-products. These carbohydrate by-products are all carried over from the fermentation process of the five individual HMO ingredients (for example, glucose, galactose, fucose, sialic acid, etc.). Acceptable limits are also included for aflatoxin M1, residual proteins and DNA, endotoxins, heavy metals, and microbiological contaminants.

Each specification parameter is measured using compendial and/or internally validated, fit-for-purpose methods. Details of the internally developed methods, HPAEC-PAD for carbohydrate analyses and qPCR for residual DNA analyses, are presented in Appendix 11 (**COMMERCIAL CONFIDENTIAL INFORMATION**). Certain analyses may be performed by external accredited laboratories their certifications are provided in Appendix 15 (**COMMERCIALLY CONFIDENTIAL INFORMATION**). Data from five batches of 5HMO-Mix show that the manufacturing process reproducibly results in a product that meets the established specifications (see Table 8). The Certificates of Analysis for these batches are provided in Appendix 16 (**COMMERCIAL CONFIDENTIAL INFORMATION**).

Table 8: Specifications and batch analysis data for Chr. Hansen's 5HMO-Mix

Parameter	Analytical Method	Specification	Batch Number				
			26115031	26126051	26131061	26102042	26142071
Physical Parameters							
Appearance (Colour)[1]	Visual	White to ivory-coloured	Complies	Complies	Complies	Complies	Complies
Appearance (Form)[1]		Spray-dried powder	Complies	Complies	Complies	Complies	Complies
Chemical Parameters							
Total HMO content	HPAEC-PAC[1]	≥ 90 (% DW)	97.5	99.5	96.4	95.9	97.7
2'-Fucosyllactose		52 ± 5 (% DW)	50.9	51.8	50.3	48.9	51.1
3-Fucosyllactose		13 ± 3 (% DW)	11.9	12.3	11.8	11.9	11.9
Lacto-*N*-tetraose		26 ± 3 (% DW)	25.9	26.3	25.6	25.8	25.7
3'-Sialyllactose		4 ± 1 (% DW)	3.8	3.8	3.7	3.7	3.8
6'-Sialyllactose		5 ± 1 (% DW)	4.9	5.3	5.1	5.7	5.3
Lactose		≤ 3 (% DW)	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
Other carbohydrates[2]		≤ 10 (% area)	2.3	2.2	2.5	3.4	4.0
Protein content[1]	Nanoquant (modified Bradford)	≤ 100 µg/g	< 10	< 10	< 10	<10	< 10
Ash[3]	ASU L 06.00-4	≤ 1.5 %	0.64	0.67	0.57	0.7	0.61
Moisture[1]	KF Titration	≤ 9.0 %	6.9	7.4	6.5	7.3	6.5
Endotoxins[4]	Ph. Eur. 2.6.14	≤ 500 EU/g	<5	<5	<5	<5	<5
Aflatoxin M1[3]	DIN EN ISO 14501	≤ 0.025 µg/kg	< 0.025	< 0.025	< 0.025	< 0.010	< 0.014

GMO residues[5]	qPCR	Negative	Negative	Negative	Negative	Negative	Negative
Heavy Metals							
Arsenic[3]	ASU L 00.00-135 – ICP-MS	≤ 0.2 mg/kg	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Cadmium[3]		≤ 0.1 mg/kg	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Lead[3]		≤ 0.02 mg/kg	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
Mercury[3]		≤ 0.1 mg/kg	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005
Microbiological Criteria							
Standard plate count[3]	ISO 4833-2	≤ 10000 cfu/g	< 10	< 10	< 10	< 10	< 10
Yeast and mold[3]	ISO 21527-2	≤ 100 cfu/g	< 20	< 20	< 20	< 20	< 20
Enterobacteriaceae[3]	ISO 21528-1	Absent/10 g	absent	absent	absent	absent	absent
Salmonella[3]	ISO 6579	Absent/100 g	absent	absent	absent	absent	absent
Cronobacter spp.[3]	ISO/TS 22964	Absent/10 g	absent	absent	absent	absent	absent

Abbreviations: cfu, colony forming units; DW, dry weight; EU, endotoxin unit; KF, Karl-Fischer; HPAEC-PAD, high performance anion exchange chromatography coupled with pulsed amperometric detection; ICP-MS, inductively coupled plasma mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation.

[1] Determined by Chr. Hansen A/S using internally validated methods. Protein LOQ = 10 µg/g. Lactose LOQ = 0.3% (DW).

[2] Carbohydrate by-products with a percent area greater than 0.5% (LOQ) are considered.

[3] Determined by the Institut für Produktqualität GmbH, which is a DIN EN ISO/IEC 17025-accredited laboratory. Ash LOQ = 0.01 %; arsenic limit of detection (LOD) = 0.05 mg/kg; cadmium LOD = 0.01 mg/kg; mercury LOD = 0.005 mg/kg; lead LOD = 0.01 ppm. For aflatoxin M1, the LOQ ranges from 0.010-0.025 µg/kg.

[4] Analyses for Batch No. 26115031, 26126051, 26131061, 26142071 were determined by an external laboratory, which is a DIN EN ISO/IEC 17025-accredited laboratory. Batch No. 26102042 was analyzed internally, also using methodologies in accordance with Ph. Eur. 2.6.14. LOQ = 5 EU/g.

[5] Determined by a DIN EN ISO/IEC 17025-accredited laboratory. LOD = 0.01% of the finished product.

Chr. Hansen's five HMOs have also been accepted for use as standalone ingredients in other jurisdictions. Following the same fermentation and downstream purification steps, the liquid HMO concentrates for 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are dried to obtain a highly purified powder that is ready for commercialisation as standalone ingredients. When marketed as standalone ingredients, the spray-dried powders containing just the individual HMOs is specified to have a purity of ≥90% for 2'-FL, ≥90% for 3-FL, ≥75% for LNT, ≥88% for 3'-SL, and ≥90% for 6'-SL on a dry weight basis (Table 9 below). Acceptable limits are also defined for residual carbohydrates that may remain in each of the HMOs when they are sold as standalone ingredients.

Table 9: Specification Limits for the Primary HMO and Residual Carbohydrates in Each of the Components of the 5HMO-Mix, when Sold as Standalone Ingredients

Parameter	**Specifications for HMOs when they are sold as standalone ingredients**				
	2'-FL	**3-FL**	**LNT**	**3'-SL**	**6'-SL**
2'-Fucosyllactose	≥ 90% DW	-	-	-	-
3-Fucosyllactose	≤ 5%	≥ 90% DW	-	-	-
Difucosyllactose	≤ 5%	-	-	-	-
Fucosylgalactose	≤ 3%	-	-	-	-
Glucose	≤ 3%	≤ 3%	-	-	-
Galactose	≤ 3%	≤ 3%	-	-	-
Fucose	≤ 3%	≤ 3%	-	-	-
Lacto-*N*-tetraose	-	-	≥ 75% DW	-	-

Lacto-*N*-triose	-	-	≤ 5%	-	-
Para-Lacto-*N*-Hexose	-	-	≤ 5%	-	-
Glucose/ galactose	-	-	≤ 5%	-	-
3'-Sialyllactose	-	-	-	≥ 88% DW	-
6'-Sialyllactose	-	-	-	-	≥ 90% DW
Sialic Acid	-	-	-	≤ 10%	≤ 10%
N-Acetylglucosamine	-	-	-	≤ 5%	≤ 5%
Lactose	≤ 5%	≤ 5%	≤ 5%	≤ 5%	≤ 5%
Sum of other carbohydrates	-	-	≤ 25%	≤ 12%	≤ 10%

To control for the presence of residual carbohydrates that are carried over from the fermentation process of the five individual HMO components (e.g., glucose, galactose, fucose, fucosylgalactose, LNT II, *para*-lacto-*N*-hexose, sialic acid, N-acetylglucosamine, etc.), the specifications for the 5HMO-Mix include an acceptable limit for "other carbohydrates" (≤10%). As indicated in Table 8 above, the levels of "other carbohydrates" across five representative batches of the 5HMO-Mix ranged from 2%-4%.

The individual specification for 2'-FL is provided in Schedule 3 S3—45 (Specification for 2'-fucosyllactose sourced from *Escherichia coli* BL21). The specifications for 3-FL, LNT, 3'-SL, and 6'-SL can be found in Table 2 of the Commission Implementing Regulation (EU):

1. 3-FL: Commission Implementing Regulation (EU) 2023/52 (Appendix 19)
2. LNT: Commission Implementing Regulation (EU) 2023/7 (Appendix 20)
3. 3'-SL: Commission Implementing Regulation (EU) 2023/113 (Appendix 21)
4. 6'-SL: Commission Implementing Regulation (EU) 2023/948 (Appendix 22)

B.6 Analytical method for detection

The analytical methods for detection of Chr. Hansen's 5HMO-Mix and other components are detailed in **Section 3.3.3 B.5** of this application.

B.7 Information on the proposed food label

No change to the infant formula product labelling requirements is anticipated due to the addition of Chr. Hansen's 5HMO-Mix to formula sold in Australia. The proposed labelling of infant formula products containing Chr. Hansen's 5HMO-Mix will be in accordance with Standard 2.9.1 of the FSANZ Food Standard Code.

C. Information related to the safety of the nutritive substances

C.1 Information on the toxicokinetics and metabolism of the nutritive substance and, if necessary, its degradation products and major metabolites

Scientific opinions published by the EFSA NDA Panel concluding that Chr. Hansen's 3-FL, LNT, 3'-SL, and 6'-SL are safe as novel foods in the EU (EFSA NDA Panel, 2022a, 2022b, 2022c, 2022d). The

conclusions made by the EFSA NDA Panel regarding the ADME profile of 3-FL, LNT, 3'-SL, and 6'-SL in these latest opinions, as well as those made in earlier opinions for these HMOs from other suppliers (EFSA NDA Panel, 2019b, 2020a, 2020b, 2021), are summarised here. In general, the EFSA NDA Panel recognises that HMOs, including 3-FL, LNT, 3'-SL, and 6'-SL (and 2'-FL) are "non-digestible carbohydrates" since they do not undergo any significant digestion in the upper gastrointestinal tract (EFSA NDA Panel, 2014). The main studies cited by the EFSA NDA Panel to support this statement within their scientific opinions for HMOs include:

- Chaturvedi et al., (2001) and Coppa et al., (2001) reported that 97% and 40%-50%, respectively, of the ingested HMOs are excreted unchanged in faeces of breastfed infants.
- HMOs, consumed as a load (a purified oligosaccharide fraction from human milk), can be fermented in the colon by intestinal microbiota (Brand-Miller et al., 1995, 1998).
- *In vitro* experiments suggest that small quantities of HMOs may be transported transcellular across the intestinal epithelium by receptor-mediated transcytosis and/or by paracellular means (Gnoth et al., 2001). Some degree of absorption and urinary excretion did occur in a study where rats were administered 2'-FL, 6'-SL, or LNnT by gavage (Vazquez et al., 2017). Similarly, evidence of systemic absorption was observed in an *in vivo* mouse micronucleus assay conducted with 3-FL, though pharmacokinetic analysis conducted as part of a 90-day oral toxicity study in rats suggests 3-FL absorption to be <1% of the daily dietary intake levels (Pitt et al., 2019).
- Data from human studies also suggest the extent of absorption to be low. Approximately 1%-2% of the ingested amounts of HMOs is excreted unchanged in the infants' urine (Goehring et al., 2014; Kunz et al., 2017; Rudloff et al., 1996, 2006).

Based on the available information, the EFSA NDA Panel came to the same general conclusions regarding the ADME profile for the HMO ingredients under consideration (EFSA NDA Panel, 2019b, 2020a, 2020b, 2021, 2022a, 2022b, 2022c, 2022d):

- Limited digestion of the HMO ingredient occurs in the gastrointestinal tract and that only small amounts are expected to be absorbed.
- Moreover, there are no indications that absorption of the HMO (3-FL, LNT, 3'-SL, 6'-SL) or other structurally related components (e.g., D-lactose), differs from that of similar components in human milk.

C.1.1 Preclinical Studies

Data from *in vitro* studies have shown that HMOs are minimally digested when incubated with digestive enzyme preparations or intestinal brush border membranes (Engfer et al., 2000; Gnoth et al., 2000). *In vitro* experiments have also mechanistically examined whether HMOs are capable of crossing the epithelium of the small intestines. Using Caco-2 human intestinal epithelial cells, it was reported that both neutral and acidic HMOs can cross the epithelial barrier, with neutral HMOs being transported across by receptor-mediated transcytosis and paracellular transport, whereas acidic HMO are absorbed *via* non-specific paracellular transport only (Gnoth et al., 2001).

Small amounts of HMOs have been detected in the serum and urine samples of rats following their oral administration. In a 13-week dietary toxicity study where Chr. Hansen's 2'-FL was administered to rats at 10% in the diet (providing approximately 7.66 and 8.72 g/kg body weight (bw)/day in males and females, respectively), mean serum levels of 2'-FL were approximately 11 µg/mL on test day 1 or 2, and approximately 2 µg/mL in test week 13. 2'-FL was also detected in the urine of the animals. Similarly, in a 90-day dietary toxicity study where 3-FL was administered to rats at 5% or 10% of 3-FL in the feed, 3-FL was detected in the serum and urine at levels that are indicative of limited systemic exposure, with absorption amounts being well below 1% of daily dietary intakes (Pitt et al., 2019). Following the administration of a single dose of 2'-FL (0.2, 1.0, 5 g/kg bw), 6'-SL (equimolecular doses equivalent to 0.2, 1.0 and 3.75 g 2'-FL/kg bw), or lacto-*N*-neotetraose (LNnT) (equimolecular doses equivalent to 0.2 and 1.0 g 2'-FL/kg bw) by gavage to rats, these HMOs were observed in serum and urine in a dose-dependent manner (Vazquez et al., 2017). In another study where a mixture of HMOs isolated from human milk was orally administered to neonatal rats by gavage at 15 g/L in a formula vehicle, the serum and urine contained detectable 3'-SL but very little amounts of other HMOs (Jantscher-Krenn et al., 2013).

A series of experiments were conducted to specifically investigate the kinetic profile using labelled 2'-FL which contained 13C in the fucose-ring (Kuntz et al., 2019). Male NMRI wild-type mice were administered a single dose of 13C-2'-FL at 1 g/kg body weight or a saline control by oral gavage, while another group of animals received 13C-2'-FL (0.2 g/kg body weight) or a saline control intravenously. To evaluate the role of the microbiota, germ-free male mice also received a single dose of 13C-2'-FL by oral gavage at 1 g/kg body weight or a saline control. After administration of the bolus oral dose, intestinal transit occurred rapidly in the wild-type animals, with maximal 13C-enrichment occurring in the small intestines at 1-hour post-dosing, and in the lower intestines at 3 hours post-administration. 2'-FL was eliminated primarily in the faeces. With respect to the absorption profile, the time-course of 13C-enrichment in the plasma and organs (liver, heart, spleen, kidney, brain) increased starting from 2 hours post-dosing (once the 2'-FL bolus has started to reach the colon), with peak accumulation reached at 5 hours. In contrast, 13C-enrichment was detected only in intestinal contents and faeces of the germ-free mice, with no significant 13C-enrichment detected in plasma, organs, or urine. Thus, these results suggest that the 13C-enrichment detected systemically after oral administration of labelled 2'-FL likely represents cleaved fucose or other metabolites of 2'-FL generated by intestinal microbes, as opposed to intact 2'-FL. In wild-type mice administered 13C-2'-FL intravenously, 13C-enrichment was detected only in the urine and not in any body compartments, which further supports the lack of 2'-FL uptake within specific organs.

C.1.2 Clinical Studies

Using a breath hydrogen test in infants, it was demonstrated that HMOs (consumed as a purified oligosaccharide fraction from human milk) was poorly digested and largely undergoes fermentation in the colon (Brand-Miller et al., 1998). In breastfed infants, it has been reported that approximately 40 to 50% (Coppa et al., 2001), and as high as 97% (Chaturvedi et al., 2001), of the ingested HMOs are excreted in the faeces. HMOs have also been detected in the plasma and urine of breastfed infants (Chaturvedi et al., 2001; Dotz et al., 2014, 2015; Goehring et al., 2014; Marriage et al., 2015; Obermeier et al., 1999; Rudloff et al., 1996, 2012; Ruhaak et al., 2014). However, the concentrations detected

were low when compared with those in human milk. In one study involving 16 infant-mother dyads, measurable amounts of 3-FL were detected in the plasma and urine samples of the breastfed infants (Goehring et al., 2014). The absolute amount of 3-FL in the samples was not quantified in this study; however, based on the absolute quantification of 2'-FL (an isomer of 3-FL) and 6'-SL, the study authors reported the relative fraction of absorbed HMOs to be low, with concentrations in plasma and urine accounting for 0.1% and 4%, respectively, of the amount ingested from breastmilk (Goehring et al., 2014). Other studies have similarly estimated that approximately 0.5 to 1.5% of the ingested HMOs are absorbed and excreted in the urine (Chaturvedi et al., 2001; Marriage et al., 2015; Obermeier et al., 1999; Rudloff et al., 1996, 2012).

Considering the structural equivalence of Chr. Hansen's HMOs to their naturally occurring counterparts in human milk (see **Section B.2.2**), it is expected that the ADME processes of the HMO ingredients in formula will mimic those of infants consuming these same HMOs through human milk.

C.2 Information from studies in animals or humans that is relevant to the toxicity of the nutritive substance and, if necessary, its degradation products and major metabolites

Chr. Hansen's 5HMO-Mix produced with genetically modified strains of *E. coli* BL21(DE3) has been evaluated in comprehensive toxicological testing, including a 13-week oral toxicity study, genotoxicity/mutagenicity assays, and a 21-day piglet feeding study. Furthermore, these toxicological studies have been conducted with Chr. Hansen's 2'-FL as a standalone preparation. These toxicological studies are described below followed by additional toxicology studies conducted with purified HMO preparations produced by other manufacturers.

C.2.1 Animal studies conducted with 5HMO-Mix

Tolerance study in neonatal piglets

The 5HMO-Mix have been evaluated in a 21-day tolerance study in neonatal piglets. The results of this study have been published (Hanlon, 2020).

Thirty-six experimentally naïve domestic two-day-old Yorkshire crossbred piglets were assigned to one of three treatment groups (n=12/group). The treatment groups received either a control diet, a diet containing 5.75 g/L of 5HMO-Mix, or a diet containing 8.0 g/L 5HMO-Mix. The control diet was Land O'Lakes Specialty Milk Replacer and it was used as the base for both of the 5HMO-Mix test diets. 5HMO-Mix contained 49.1% 2'-FL, 10.4% 3-FL, 19.9% LNT, 3.5% 3'-SL, and 4.2% 6'-SL on a dry weight basis. The endpoints that were evaluated included mortality, clinical observations, body weight, feed consumption, feed efficiency, compound consumption, clinical pathology parameters (haematology, coagulation, clinical chemistry, and urinalysis), gross necropsy findings, organ weights, and histopathologic examinations.

There were no treatment-related differences in body weight, food consumption, or feed efficiency between groups. Furthermore, there were no differences in haematology, clinical chemistry, or urinalysis parameters on Study Day 7 and Study Day 21 that could be attributed to 5HMO-Mix, nor were there any findings in organ weights, or macroscopic and microscopic inspection of tissues that

could be attributed to 5HMO-Mix. Although increased cecum weights in males and females at ≥5.75 g/L, increased colon weights in males at ≥5.75 g/L, and decreased rectum weights in males and females at 8.0 g/L were observed, these changes were not considered adverse as there were no microscopic correlates. Except for one male piglet in the 8.0 g/L dosing group, which was euthanized on day 7 for humane reasons, all of the remaining animals survived until the scheduled study termination on day 22. The clinical and veterinary observations of the male piglet in the 8.0 g/L dosing group that was euthanized included yellow discoloured faeces, thin body condition, unkempt appearance, generalized muscle wasting, and lateral recumbency. Additionally, *E. coli* was detected in a faecal culture of that euthanized male piglet. Based on the presence of *E. coli* in the faeces and the constellation of observations, the unscheduled death/euthanasia of the one male in the 8.0 g/L treatment group was determined to be not related to the administration of 5HMO-Mix, but rather due to an underlying bacterial infection that was likely obtained at the farm prior to enrolment in the study.

Together, these results indicate that daily dietary administration of 5HMO-Mix to neonatal piglets for 3 weeks, at concentrations up to 8.0 g/L in milk replacer, was well-tolerated, did not produce adverse effects on growth and development. This dosage corresponds to calculated intakes of the 5HMO-Mix at 3.6 and 3.7 g/kg bw/day in males and females, respectively. Given the composition of the 5HMO-Mix test article (49.1% 2'-FL, 10.4% 3-FL, 19.9% LNT, 3.5% 3'-SL, and 4.2% 6'-SL on a dry weight basis), this corresponds to intakes of approximately 1.8 g 2'-FL/kg bw/day, 0.4 g 3-FL/kg bw/day, 0.7 g LNT/kg bw/day, 0.1 g 3'-SL/kg bw/day, and 0.2 g 6'-SL/kg bw/day).

Sub-chronic oral toxicity study

Chr. Hansen's 5HMO-Mix has been evaluated in a 13-week dietary toxicity study conducted in compliance with OECD Guideline No. 408 and Good Laboratory Practice (GLP). The results of this study have been published Parschat et al. (2020), and the full study report is provided in Appendix 17 (**COMMERCIALLY CONFIDENTIAL INFORMATION**). The test article consisted of 2'-FL (47.1% dry weight), 3-FL (16.0% dry weight), LNT (23.7% dry weight), 3'-SL (4.1% dry weight), 6'-SL (4.0% dry weight), and other minor carbohydrates accounting for the remainder (5.1% dry weight).

Administration of 5HMO-Mix at 10% in the diet of female CD rats was well tolerated in an initial 7-day pilot study (see Appendix 17 (**COMMERCIALLY CONFIDENTIAL INFORMATION**)). Thus, this concentration was selected for the sub-chronic dietary toxicity study. In this study, CD rats (10/sex/group) were fed a control diet, or the same diet containing 10% of the 5HMO-Mix *ad libitum* for 91 days. No test item-related changes were observed for animal behaviour or external appearance, nor were there any relevant changes with respect to detailed clinical observations, ophthalmological examinations, neurological parameters, body weight, body weight gain, body weight at autopsy, food and drinking water consumption, haematological, clinical chemistry, urinalysis, macroscopic inspection at necropsy, the majority relative and absolute organ weights, or the myeloid/erythroid ratio in the bone marrow. Although some statistically significant changes were noted in body temperature, motility, neutrophilic granulocytes, selected clinical chemistry parameters, absolute and relative organ weights (brain and kidneys), and the specific gravity of the urine in the 5HMO-Mix-treated animals, all deviations were limited to one sex, within the historical range for the laboratory, generally below 20%, and deemed to be not 5HMO-Mix-related. Additionally, the histopathological

examination revealed no test item-related morphological changes at the end of the 91-day treatment period. A mild increase in the incidence of hepatocellular lipid was limited to male rats in the test group, which again was not consistent with other study findings, and therefore not considered related to the 5HMO-Mix.

Overall, the NOAEL for 5HMO-Mix in this study was concluded to be 10% in the diet, which is equivalent to intakes of 5HMO-Mix at 5.67 g/kg bw/day for males and 6.97 g/kg bw/day for females. Based on their concentrations within the 5HMO-Mix, the intakes of the individual HMOs in this study are as follows for males and females, respectively: 2.67 and 3.28 g/kg bw/day for 2'-FL; 0.91 and 1.12 g/kg bw/day for 3-FL; 1.34 and 1.65 g/kg bw/day for LNT: 0.23 and 0.29 g/kg bw/day for 3'-SL; and 0.23 and 0.28 g/kg bw/day for 6'-SL.

Genotoxicity/ mutagenicity

Chr. Hansen's 5HMO-Mix has been evaluated in a bacterial reverse mutation assay and an *in vitro* micronucleus assay. The results of these studies have been published by Parschat et al. (2020), and the full study reports are provided in Appendix 17 (**COMMERCIALLY CONFIDENTIAL INFORMATION**). The 5HMO-Mix test article was identical to those used for the 13-week dietary toxicity study, comprising 2'-FL (47.1% dry weight), 3-FL (16.0% dry weight), LNT (23.7% dry weight), 3'-SL (4.1% dry weight), 6'-SL (4.0% dry weight), and other minor carbohydrates accounting for the remainder (5.1% dry weight).

In the bacterial reverse mutation assay, which was compliant with OECD Guideline No. 471 and GLP, two independent experiments were conducted with strains of *Salmonella* Typhimurium (TA98, TA100, TA102, TA1535, and TA1537). Each were conducted in triplicates, with and without metabolic activation. The first experiment was conducted as a plate incorporation test and the second as a preincubation test. The 5HMO-Mix was applied at concentrations of 5.0, 10.0, 31.6, 100, 316 or 600 mg/plate. No cytotoxicity or mutagenicity were noted in any of test strains at the highest concentrations tested in either the plate incorporation or preincubation tests. Therefore, it can be concluded that 5HMO-Mix was not mutagenic under the conditions of the assay.

The *in vitro* micronucleus assay was conducted in accordance with OECD Guideline No. 487 and GLP. Human peripheral blood lymphocytes were incubated in medium containing the 5HMO-Mix at concentrations of 7.5, 15, 30, and 60 mg/mL for 4 or 24 hours, in the presence and absence of metabolic activation. No chromosomal damage was observed with 5HMO-Mix under the conditions tested, and 5HMO-Mix was concluded to be not genotoxic based on the results of this assay.

C.2.2 Animal studies conducted with Chr. Hansen's 2'-FL

Tolerance study in neonatal piglets

In brief, a total of 27 male and 21 female Yorkshire piglets were administered a standard milk replacer (ProNurse® Specialty Milk Replacer), or the same milk replacer supplemented with 2'-FL at 200 mg, 500 mg or 2000 mg/L, starting from 2 days after birth for 21 days (Hanlon & Thorsrud, 2014).

All piglets survived to scheduled necropsy on Day 22. There were no reported dose-responsive adverse clinical findings during the dosing period. Both male and female piglets showed good growth based on body weight gain and feed efficiency. There were no treatment-related adverse effects on the clinical pathology parameters evaluated, including haematology, clinical chemistry, coagulation and urinalysis. There were also no treatment-related adverse macroscopic and histopathologic changes. These results indicate that daily dietary administration of 2'-FL to neonatal piglets for 3 weeks following birth, at concentrations up to 2,000 mg/L in milk replacer, was well tolerated and did not produce any adverse treatment-related effects on growth and development. The intake of 2'-FL was calculated to be 291.74 and 298.99 mg/kg bw/day in males and females, respectively.

Sub-chronic oral toxicity study

Chr. Hansen's 2'-FL has been evaluated in a 90-day dietary toxicity study which was conducted in accordance with OECD Guideline No. 408 and GLP. No treatment-related adverse effects were observed in the 7-day pilot study, or the 90-day dietary toxicity study where Crl:CD(SD) rats were fed diets containing 10% 2'-FL. Accordingly, the NOAEL for 2'-FL from this study was derived as 10% in the diet, which equated to approximately 7.66 g/kg bw/day in males and 8.72 g/kg bw/day in females. These studies are unpublished.

Genotoxicity/ mutagenicity

Chr. Hansen's 2'-FL was demonstrated to be non-mutagenic when tested in a bacterial reverse mutation assay compliant with OECD Guideline No. 471 and GLP. Additionally, no evidence of genotoxicity was observed when Chr. Hansen's 2'-FL was tested using an *in vivo* micronucleus assay in Crl:CD(SD) rats that was compliant with OECD Guideline No. 474 and GLP. These studies are unpublished.

C.2.3 Animal studies conducted using purified HMO preparations produced by other manufacturers

Repeated-dose toxicology studies

Purified preparations of HMOs (2'-FL, 3-FL, LNT, 3'-SL, 6'-SL) produced by other manufacturers, either by microbial fermentation with a genetically modified strain of *E. coli* K12 or chemical/enzymatic synthesis, have also been evaluated in toxicological studies. A summary of these studies is presented in the table below. For comparison, the studies conducted with Chr. Hansen's 2'-FL and 5HMO-Mix are also included.

Although the studies were conducted with HMOs produced by other methods, the data help support those purified preparations of HMOs, including those derived from microbial fermentation with safe and well-characterized production strains, are without adverse effects.

Table 10: Animal toxicity studies conducted with HMOs

Test Article	Reference[1]	Manufacturer and Production Method	Study Type	Doses (g/kg bw/day) or Dietary Concentrations	NOAEL[2]
Rodent studies					
2'-FL	Coulet et al., 2014	Glycom A/S (Chemical synthesis)	14-day DRF study in rats	0, 2, 5, 7.5	5 g/kg bw/day
			90-day oral toxicity study in neonatal rats (adapted OECD method)	0, 2, 5, 6	
	Unpublished	Chr. Hansen (Fermentation)	7-day pilot tolerance study in rats	0, 10% in diet	10% in diet, corresponding to 7.66 g/kg bw/day (males); 8.72 g/kg bw/day (females)
			90-day dietary toxicity study in rats (OECD)	0, 10% in diet	
	Unpublished (Penard, 2015 – see GRN No. 650)	Glycom A/S (Fermentation)	90-day oral toxicity study in neonatal rats (adapted OECD method)	0, 2, 4, 5	5 g/kg bw/day
	Van Berlo et al., 2018	Friesland Campina Domo (Fermentation)	90-day dietary toxicity study in rats (OECD)	0, 3, 6, 10% in the diet	10% in diet, corresponding to 7.25 g/kg bw/day (males); 7.76 g/kg bw/day (females)
	Unpublished (Case and Yoon, 2020 – see GRN No. 932)	Advanced Protein Technologies Corp. (Fermentation)	Acute oral toxicity study in rats	0, 2.5, 5, 7.5	LD_{50} >7.5 g/kg bw
			90-day oral toxicity study in rats (OECD)	0, 2.5, 5, 7.5	7.5 g/kg bw/day
3-FL	Pitt et al., 2019	DuPont Nutrition and Biosciences (Fermentation)	Acute oral toxicity study in rats (OECD)	0, 5	LD_{50} >5 g/kg bw
			90-day dietary toxicity study in rats (OECD)	0, 5, 10% in diet	10% in diet, corresponding to 5.98 g/kg bw/day (males); 7.27 g/kg bw/day (females)
	Phipps et al., 2022	Glycom A/S (Fermentation)	14-day DRF study in rats	0, 3, 4	4 g/kg bw/day[4]
			90-day oral toxicity study in neonatal rats (adapted OECD method)	0, 1, 2, 4	
LNT	Phipps et al., 2018b	Glycom A/S (Fermentation)	14-day DRF study in rats	0, 3.25, 4	4 g/kg bw/day[4]

Test Article	Reference[1]	Manufacturer and Production Method	Study Type	Doses (g/kg bw/day) or Dietary Concentrations	NOAEL[2]
			90-day oral toxicity study in neonatal rats (adapted OECD method)	0, 1, 2.5, 4	
3'-SL	Kim et al., 2018	GeneChem, Inc. (Enzymatic Synthesis)	Acute oral toxicity study in rats (OECD)	0, 5, 10, 15, 20	LD_{50} >20 g/kg bw
			28-day oral toxicity study in rats (OECD)	0, 0.5, 1, 2	2 g/kg bw/day
			90-day oral toxicity study in rats (OECD)	0, 0.5, 1, 2	2 g/kg bw/day
			Dose escalation single-dose toxicity study in Beagle dogs (GLP)	0, 0.5, 1, 2	2 g/kg bw/day
	Phipps et al., 2019a	Glycom A/S (Fermentation)	14-day DRF study in rats	0, 4, 5	5 g/kg bw/day
			study in neonatal rats (adapted OECD method)	0, 1, 3, 5	
6'-SL	Gurung et al., 2018a	Glycom A/S (Enzymatic synthesis)	Acute oral toxicity study in rats (FDA Redbook)	0, 5, 10, 15, 20	LD_{50} >20 g/kg bw
			13-week oral toxicity study (FDA Redbook)	0, 1, 2.5, 5	5 g/kg bw/day
	Phipps et al., 2019b	Glycom A/S (Fermentation)	14-day DRF study in rats	0, 4, 5	5 g/kg bw/day
			90-day oral toxicity study in neonatal rats (adapted OECD method)	0, 1, 3, 5	
2'-FL/ DFL5	Unpublished (Flaxmer, 2017 – see GRN No. 815)	Glycom A/S (Fermentation)	14-day DRF study in rats	0, 4, 5	5 g/kg bw/day
	(Phipps et al., 2018a)	Glycom A/S (Fermentation)	90-day oral toxicity study in neonatal rats (adapted OECD method)	0, 1, 3, 5	
5HMO-Mix	Parschat et al., 2020	Chr. Hansen[3] Each HMO is produced by separate fermentation processes and then blended.	7-day pilot tolerance study in rats	0, 10% in diet	10% in diet, corresponding to 5.67 g/kg bw/day (males); 6.97 g/kg bw/day (females)
			90-day dietary toxicity study in rats (OECD-compliant)	0, 10% in diet	

Test Article	Reference[1]	Manufacturer and Production Method	Study Type	Doses (g/kg bw/day) or Dietary Concentrations	NOAEL[2]
Neonatal piglet tolerance studies					
2'-FL	Hanlon & Thorsrud, 2014	Chr. Hansen[3] (Fermentation)	21-day neonatal piglet tolerance study	0, 0.2, 0.5, 2 g/L in milk replacer	2 g/L in milk replacer, corresponding to ~0.29 g/kg bw/day
3-SL	Monaco et al., 2019	GeneChem, Inc. (Enzymatic synthesis)	21-day neonatal piglet tolerance study	0, 0.14, 0.2, 0.5 g/L in milk replacer	0.5 g/L in milk replacer (~0.18 g/kg bw/day)[6]
6'-SL	Monaco et al., 2020	GeneChem, Inc. (Enzymatic synthesis)	21-day neonatal piglet tolerance study	0, 0.3, 0.6, 1.2 g/L in milk replacer	1.2 g/L in milk replacer (~0.4 g/kg bw/day)[7]
SL mixture[8]	Monaco et al., 2018	Arla Foods Ingredients Group P/S	Neonatal piglet study from PND 2 until 32/33 day of life (~4 weeks)	0, 0.13, 0.38, 0.76 g/L in milk replacer	0.76 g/L in milk replacer (~0.25 g/kg bw/day)[8]
5HMO-Mix	Hanlon, 2020	Chr. Hansen[3] Each HMO is produced by separate fermentation processes and then blended.	21-day neonatal piglet tolerance study	0, 5.75, 8 g/L in milk replacer	8 g/L of total HMOs in milk replacer (~3.6 g/kg bw/day)

Abbreviations: bw = body weight; DFL, difucosyllactose; DRF, dose-range finding study; LD50, median lethal dose; LNFP-I, lacto-N-fucopentaose I; NOAEL = no-observed-adverse-effect level; NR, not reported; PND, postnatal day.

[1] For unpublished studies conducted by other manufacturers, details of the methodologies and results are publicly available in the GRAS notices that have been filed with the U.S. FDA for that preparation.

[2] All of the NOAELs in this table represent the highest dose tested by the study authors, except for the Coulet et al., (2014) study. In that study, the NOAEL was conservatively set at the mid-dose of 5 g/kg bw/day. Some effects (e.g., colored/liquid feces, erythema in the urogenital region during the first 2 weeks of treatment, hypersalivation, a lower initial body weight gain, and unexplained mortalities) were observed in the animals of the highest 2'-FL dose group (6 g/kg bw/day), though such effects were also observed in a reference control group administered 6 g/kg bw/day of FOS.

[3] Previously known as Jennewein Biotechnologie GmbH.

[4] Represents the maximum feasible dose that could be tested due to viscosity of the test article.

[5] 2'-FL and DFL are produced together from microbial fermentation with a single strain. The test article contained 82.5% (w/w) of 2'-FL and 9.70% (w/w) of DFL.

[6] The intake of 3'-SL on a body weight basis was not reported by the study authors. However, it was indicated that the formula was reconstituted fresh daily and delivered daily to the piglets at 360 mL/kg bw from day 6 onwards. Thus, the intake of 3'-SL at the 0.5 g/L concentration can be approximately calculated as 0.18 g/kg bw/day.

[7] The intake of 6'-SL on a body weight basis was not reported by the study authors. However, it was reported that the formula was reconstituted fresh daily and delivered daily to the piglets at 330 mL/kg bw from day 6 onwards. Thus, the intake of 6'-SL at the 1.2 g/L concentration can be approximately calculated as 0.4 g/kg bw/day.

[8] Test article is bovine-derived modified whey enriched with SL, presumably 3'-SL and 6'-SL (Lacprodan SAL-10). The intake of SL on a body weight basis was not reported by the study authors. However, it was reported that the formula was reconstituted fresh daily and delivered daily to the piglets at 325 mL/kg bw from day 8 onwards. Thus, the intake of SL at the 0.76 g/L concentration can be approximately calculated as 0.25 g/kg bw/day.

Genotoxicity/ mutagenicity

In addition to the studies conducted with Chr. Hansen's HMOs, the absence of genotoxicity/mutagenicity have been demonstrated for purified preparations of 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL produced by other manufacturers. The results of these studies are summarised in the table below.

Table 11: Summary of genotoxicity/ mutagenicity assays conducted with HMOs

Test Article	Reference[1]	Manufacturer and Production Method	Study Type	Conclusions
2'-FL	Coulet et al., 2014	Glycom A/S (Chemical synthesis)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro mammalian cell gene mutation assay (OECD)	Not mutagenic
	Unpublished (Verbaan, 2015a – see GRN No. 650)	Glycom A/S (Chemical synthesis)	*In vitro* micronucleus test (OECD)	Not clastogenic or aneugenic
	Unpublished	Chr. Hansen[2] (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vivo micronucleus test in rats (OECD)	Not genotoxic
	Unpublished (Verspeek-Rip, 2015 – see GRN No. 650)	Glycom A/S (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
	Unpublished (Verbaan, 2015b – see GRN No. 650)	Glycom A/S (Fermentation	*In vitro* micronucleus test (OECD)	Not clastogenic or aneugenic
	Van Berlo et al., 2018	Friesland Campina Domo (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro micronucleus test (OECD)	Not clastogenic or aneugenic
	Unpublished (Case and Yoon, 2020 – see GRN No. 392)	Advanced Protein Technologies Corp. (Fermentation)	Bacterial reverse mutation test	Not mutagenic
			In vitro chromosome aberration test	Not clastogenic or aneugenic
			In vivo micronucleus test in mice	Not genotoxic
3-FL	Pitt et al., 2019	DuPoint Nurition and Biosciences (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro chromosome aberration test (OECD)	Not clastogenic or aneugenic
			In vitro micronucleus test (OECD)	Not genotoxic
			In vivo micronucleus test in mice (OECD)	

Test Article	Reference[1]	Manufacturer and Production Method	Study Type	Conclusions
	Phipps et al., 2022	Glycom A/S (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro micronucleus test (OECD)	Not clastogenic or aneugenic
LNT	Phipps et al., 2018b	Glycom A/S (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro micronucleus test (OECD)	Not clastogenic or aneugenic
3-SL	Kim et al., 2018	GeneChem, Inc. (Enzymatic synthesis)	Bacterial reverse mutation test	Not mutagenic
			In vitro chromosome aberration test	Not clastogenic or aneugenic
			In vivo micronucleus test in mice	Not genotoxic
	Phipps et al., 2019a	Glycom A/S (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro micronucleus test (OECD)	Not clastogenic or aneugenic
6'-SL	Gurung et al., 2018a	GeneChem, Inc. (Enzymatic synthesis)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro chromosome aberration test (OECD)	Not clastogenic or aneugenic
			In vitro micronucleus test (OECD)	Not genotoxic
	Phipps et al., 2019b	Glycom A/S (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro micronucleus test (OECD)	Not clastogenic or aneugenic
2'-FL/ DFL[4]	Phipps et al., 2018a	Glycom A/S (Fermentation)	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro micronucleus test (OECD)	Not mutagenic
5HMO-Mix	Parschat et al., 2020	Chr. Hansen[2] Each HMO is produced by separate fermentation processes and then blended.	Bacterial reverse mutation test (OECD)	Not mutagenic
			In vitro micronucleus test (OECD)	Not clastogenic or aneugenic

C.2.4 Human clinical studies conducted with 5HMO-Mix

Lasekan et al., 2022 (NCT04105686)

Abbott Nutrition completed a growth monitoring study (ClinicalTrials.gov identifier: NCT04105686), which compared the growth of infants receiving a milk-based experimental formula that contained a blend of five HMOs at 5.75 g/L to the growth of infants receiving the same formula without HMOs (control). The base formula employed in this study is compliant with the compositional requirements

for infant formula in Canada, as defined in Section B.25.054 of the *Food and Drug Regulations* (Lasekan et al., 2022).

The study was a 16-week randomised, controlled, blinded growth and tolerance study. In addition to the test and control formula groups, a human milk-fed reference group (HM) was also included. Healthy term infants (n=366) were enrolled in the study between birth and 14 days of age. The primary variable of the study was weight gain per day from 14 to 119 days of age of infants in the two formula groups. Values at days 14, 38, 42, 56, 84 and 119 of life were used for the primary analysis. Results comparing the two infant formula groups to each other and to a human milk reference group for weight gain per day from 14 to 119 days of age indicated that there were no statistically significant differences in growth. Sensitivity analysis likewise showed no statistically significant differences among the three groups. Furthermore, the experimental formula was non-inferior to control using a non-inferiority margin of 3 g/day in primary and sensitivity analyses. Both formulas were well tolerated.

In conclusion, this clinical study demonstrated that a formula containing 5.75g/L of HMOs was safe, well tolerated and supported normal growth.

Parschat et al., 2021 (NCT03513744)

A multi-centred, randomised, double-blinded, controlled, parallel group clinical study was conducted to evaluate the safety and tolerability of the 5HMO-Mix (ClinicalTrials.gov identifier: NCT03513744). The results of this study have been published (Parschat et al., 2021).

Healthy term infants ≤14 days of age were randomised to receive exclusive feeding with an infant formula containing 5HMO-Mix (n=113) or a control infant formula (n=112) as a reference control (n=116), for 4 months. A group of exclusively breastfed infants (BM) was also included as a reference control (n=116). The 5HMO-Mix provided 2.99 g/L 2'-FL, 0.75 g/L 3-FL, 1.5 g/L LNT, 0.23 g/L 3'-SL and 0.28 g/L 6'-SL, which was achieved by partially replacing some of the carbohydrates (*i.e.*, maltodextrin) in the standard formula.

There were no statistically significant differences in body weight, length, or head circumference gain between the two formula groups, and formula supplemented with 5HMO-Mix was considered non-inferior to the control formula with respect to mean daily body weight gain. The total incidence of adverse events (AEs) and serious AEs were comparable across all three groups. Overall, the addition of 5HMO-Mix into infant formula is considered safe and well-tolerated.

C.2.5 Human clinical studies conducted with individual HMOs

Studies conducted in infants

A number of clinical studies have been conducted to evaluate the safety and tolerability of infant formula supplemented with 2'-FL, either on its own, in combination with LNnT (which is an isomer of LNT), or with other non-digestible carbohydrates such as galacto-oligosaccharides (GOS), or fructo-oligosaccharides (FOS) (Berger et al., 2020; Dogra et al., 2021; Goehring et al., 2016; Kajzer et al., 2016; Marriage et al., 2015; Nowak-Wegrzyn et al., 2019; Puccio et al., 2017; Ramirez-Farias et al., 2021;

Riechmann et al., 2020; Storm et al., 2019; Vandenplas et al., 2020, 2022). Administration of formulas supplemented with 2'-FL in these studies was safe and well-tolerated by infants.

Several clinical studies have also been conducted using infant formula supplemented with bovine milk-derived oligosaccharides (BMOs) (Castanet et al., 2020; Cooper et al., 2016; Estorninos et al., 2021, 2022; Meli et al., 2014; Radke et al., 2017; Simeoni et al., 2016). In these studies, the BMOs mixture was added at levels providing total oligosaccharide concentrations ranging from 6 to 10 g/L in the reconstituted formula. The BMOs mixture contained oligosaccharides such as 3'-SL, 6'-SL, and GOS in an unspecified amount. Although the effects of 3'-SL and 6'-SL cannot be independently assessed in these studies, it was reported that infant formula supplemented with BMOs was generally well tolerated and supported normal growth.

Studies conducted in toddlers

A randomized, double-blind, controlled clinical study was recently published that evaluated the effects of a "young child formula" (YCF) supplementation on the incidence of gastrointestinal and upper respiratory infections among children aged 1 to 2.5 years (Leung et al., 2020). The children (n=146) received 1 of 4 interventions for 6 months: a standard milk-based formula (YCF-ref); a milk formula containing 3 g/L of 2'-FL, immunoglobins (1 g/L), lactoferrin (1.7 g/L), TGF-beta (15 µg/L), and milk fat (2.5 g/100 mL) (termed YCF-A); a milk formula that is the same as YCF-A but with lower levels of immunoglobulins (0.1 g/L), lactoferrin (0.1 g/L), and no added 2'-FL or milk fat (termed YCF-B); or a milk formula that is the same as YCF-ref but with 3 g/L of 2'-FL (termed YCF-C). All 4 formulas also contained 4 g/L of GOS. The children consumed two 200 mL servings of the YCF daily (400 mL/day) for 6 months. No "remarkable between-group differences" were observed in anthropometric parameters, assessed as the z-scores for weight-for-age, height-for-age, and weight-for-height. The incidence of adverse events and serious adverse events were similar across groups, with no reported cases of product-related events as judged by investigators and confirmed by an independent data safety monitoring board. The study authors concluded all the YCFs tested were considered safe and supported normal growth.

Studies conducted in older children and adults

Clinical studies have also evaluated the effects of 2'-FL supplementation in older children and in adults. Supplementation with 2'-FL, either alone or as a 4:1 mixture with LNnT, at 4.5 g/day for 8 weeks was safe and well tolerated in overweight/obese children between the ages of 6 to 12 years old (Fonvig et al., 2021). In one randomized, placebo-controlled, double-blind, parallel study designed to assess safety and tolerability, ingestion of up to 20 g/day of either 2'-FL, LNnT, or a combination of 2'-FL and LNnT at a 2:1 ratio, was concluded to be well tolerated in healthy adults (Elison et al., 2016). Supplementation with 2'-FL was also reported to be well tolerated in adults with gastrointestinal conditions (e.g., irritable bowel syndrome) (Iribarren et al., 2020; Palsson et al., 2020; Ryan et al., 2021). Additionally, several studies have been conducted where adults were administered 3'-SL (Gurung et al., 2018b; Opekun et al., 1999; Parente et al., 2003; Rasko et al., 2000), or 6'-SL (Kim et al., 2022). BMO mixtures containing sialylated oligosaccharides have also been evaluated in children (Sanctuary et al., 2019) and adults (Smilowitz et al., 2017; Westreich et al., 2020). Although these

studies have limited relevance in supporting the use of Chr. Hansen's HMOs in infant formula and toddler formula, it is noteworthy that administration of these individual HMOs beyond infancy was generally without adverse effects.

C.3 Safety assessment reports prepared by international agencies or other national government agencies, if available

Manufactured HMO ingredients have regulatory clearance for addition to infant formula and toddler formulas, as well as a range of other food products, in a number of jurisdictions. As examples, the regulatory approvals for 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL in Australia, the United States (U.S.) and European Union (EU) are presented in the subsections below.

C.3.1 Australia

At present, FSANZ permits the addition of the following HMOs as per Schedule 29 of the Food Code:

- 2'-FL at a maximum level of 2.4 g/L or 96 mg/100kJ
- LNT at a maximum level of 32 mg/100kJ
- 3'-SL at a maximum level of 8mg/100kJ
- 6'-SL at a maximum level of 16 mg/100kJ

Furthermore, FSANZ has approved or is in the process of reviewing the following applications to amend the Food Standards Code, as listed in Table 12 below.

Table 12: List of applications made to FSANZ with regards to HMOs

Application No.	HMO Ingredient(s)	Applicant	Approval Date
A1155	2'-FL, LNnT	Glycom A/S	December 2019
A1190	2'-FL	Jennewein Biotechnologie GmbH[1]	November 2021
A1233	2'-FL	FrieslandCampina Ingredients	May 2022
A1251	2'-FL with galacto-oligosaccharides and/or inulin-type fructans	Nutricia Australia Pty Ltd & Chr. Hansen A/S[1]	December 2022
A1265	2'-FL/DFL, LNT, 6'-SL, 3'-SL	Glycom A/S	September 2023
A1277	2'-FL	Inbiose N.V. Belgium	March 2024
A1308	2'-FL	Kyowa Hakko Bio Co., Ltd	Application on-going
A1324	3-FL	Glycom A/S	Application on-going
[1] Currently part of Novonesis group.			

In Australia, 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are listed in the Therapeutic Goods (Permissible Ingredients) Determination (No. 1) 2025 as active ingredients for use in complementary medicines. A summary of the ingredient name, purpose, and maximum limits are described in Table 13 below.

Table 13: HMOs permitted in the Therapeutic Goods (Permissible Ingredients) Determination (No. 1) 2025

Ingredient name	Purpose	Maximum Limits
2'-fucosyllactose	Active ingredient	The maximum recommended daily dose of the medicine must not provide more than: a. 12 g of 2'-fucosyllactose to individuals aged 13 years and older; b. 4 g of 2'-fucosyllactose to individuals aged between 1 and 12 years (inclusive); and c. 1.2 g of 2'-fucosyllactose to individuals aged between 1 and 11 months (inclusive). 2'-fucosyllactose is not permitted for use in children under the age of 1 month.
3-fucosyllactose	Active ingredient	The maximum recommended daily dose of the medicine must not provide more than: a. 2 g of 3-fucosyllactose to individuals aged 0 to 3 years (inclusive); and b. 5 g of 3-fucosyllactose to individuals aged 4 years and older.
Lacto-N-tetraose	Active ingredient	The maximum recommended daily dose of the medicine must not provide more than: a) 2 g of lacto-N-tetraose to individuals aged 1 year and older; and b) 0.6 g of lacto-N-tetraose to individuals aged more than 6 months to 11 months (inclusive); and c) 0.8 g of lacto-N-tetraose to individuals aged up to 6 months (inclusive).
3'-sialyllactose sodium	Active ingredient	The maximum recommended daily dose of the medicine must not provide more than: a. 0.2 g 3'-sialyllactose sodium in infants under 12 months; b. 0.15 g 3'-sialyllactose sodium in children aged 12-35 months; or c. 0.5 g 3'-sialyllactose sodium in individuals aged 3 years and older.
6'-sialyllactose sodium	Active ingredient	The maximum recommended daily dose of the medicine must not provide more than: a. 0.4 g 6'-sialyllactose sodium in infants under 12 months; b. 0.3 g 6'-sialyllactose sodium in children aged 12-35 months; or c. 1.0 g 6'-sialyllactose sodium in individuals aged 3 years and older.

C.3.2 United States

The five individual HMOs produced by Chr. Hansen using genetically modified strains of *E. coli* BL21(DE3) have been concluded Generally Recognized as Safe (GRAS) for their intended uses in formula in the U.S., which have been notified to the Food and Drug Administration (FDA) and filed with "no questions". The GRAS status of HMOs produced by other manufacturers have also been notified to the FDA and filed with "no questions". A summary of these GRAS notices, including the use levels for HMOs in infant formula and toddler formula, is listed in Table 14 below.

Table 14: GRAS notices for the five HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL) filed with "No Questions" by the U.S. FDA

HMO	GRN No.[1]	Notifier	Accepted Use Levels (As Consumed)		
			Infant Formula (0-12 months)[2]	Toddler Formula (1-3 years)	Other Food Categories[3]
2'-FL	546	Glycom A/S	2.4 g/L	2.4 g/L	Yes
	571	Chr. Hansen[4]	2.0 g/L	2.0 g/L	Not included[4]
	650	Glycom A/S	2.4 g/L	2.4 g/L	Yes
	735	Friesland Campina Domo B.V., Glycosyn LLC	2.4 g/L	2.4 g/L	Yes
	749	DuPont Nutrition and Health	2.4 g/L	2.4 g/L	See GRN 897
	852	BASF	2.4 g/L	2.4 g/L	Yes
	897	DuPont Nutrition and Health	2.4 g/L	2.4 g/L	Yes
	929	Chr. Hansen[4]	2.0 g/L (exempt HA term IF)	2.0 g/L (HA formula)	Not included[4]
	932	Advanced Protein Technologies Corp.	2.4 g/L	2.4 g/L	Yes
	1014	Chr. Hansen[4]	-	2.4 g/L	Yes
	1034	Glycom A/S	2.4 g/L (exempt HA term IF)	-	-
	1051	Kyowa Hakko	2.4 g/L	2.4 g/L	Yes
	1060	Glycom A/S	2.4 g/L	2.4 g/L	Yes
	1091	Inbiose N.V.	2.4 g/L	2.4 g/L	Yes
3-FL	925	Chr. Hansen[4]	0.44 g/L	Not included[4]	Not included[4]
	951	DuPont Nutrition and Health	0.44 g/L	0.44 g/L	Yes
	1037	Glycom A/S	0.75 g/L	0.90 g/L	Yes
	1099	Chr. Hansen[4]	0.9 g/L	1.2 g/L	Yes
LNT	833	Glycom A/S	0.8 g/L	0.6 g/L	Yes
	923	Chr. Hansen[4]	0.8 g/L	Not included[4]	Not included[4]
	1017	Chr. Hansen[4]	-	0.8 g/L	Yes
	1068	Inbiose N.V.	0.8 g/L	0.6 g/L	Yes
3'-SL	766	GeneChem Inc.	0.24 g/L	0.25 g/L	Yes
	880	Glycom A/S	0.20 g/L	0.15 g/L	Yes
	921	Chr. Hansen[4]	0.28 g/L	Not included[4]	Not included[4]
	1015	Chr. Hansen[4]	-	0.28 g/L	Yes
	1052	Kyowa Hakko	0.24 g/L	0.24 g/L	Yes
	1074	Inbiose N.V.	0.28 g/L	0.28 g/L	Yes
6'-SL	881	Glycom A/S	0.4 g/L	0.3 g/L	Yes
	922	Chr. Hansen[4]	0.4 g/L	Not included[4]	Not included[4]
	1016	Chr. Hansen[4]	-	0.4 g/L	Yes
	1053	Kyowa Hakko	0.4 g/L	0.5 g/L	Yes
	1075	Inbiose N.V.	0.4 g/L	0.3 g/L	Yes

HMO	GRN No.[1]	Notifier	Accepted Use Levels (As Consumed)		
			Infant Formula (0-12 months)[2]	Toddler Formula (1-3 years)	Other Food Categories[3]
Abbreviations: FDA, Food and Drug Administration; GRAS, Generally Recognised as Safe; HA, hypoallergenic; IF, infant formula; U.S., United States. [1] Only GRAS notices that have received a "no questions" response from the U.S. FDA (as of April 2022) is included in this table. Of note, Chr. Hansen's LNnT produced with *E. coli* BL21(DE3), and LNnT from other manufacturers, also have GRAS status in the U.S. for use in infant formula (GRN No. 919, 659, 547). [2] Refers to non-exempt infant formula for term infants, unless otherwise stated. [3] Details on the specific food uses are available in the corresponding GRAS notice. [4] Previously known as Jennewein Biotechnologie GmbH.					

C.3.3 European Union

In the EU, 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL produced by microbial fermentation are authorised for use as novel foods in infant formula, toddler formula, and a broad range of other food categories, as summarised in Table 15 below.

Table 15: Authorised food uses and use levels for HMOs in the European Union

Specified Food Category	Qualifiers	Maximum Use Levels[1,2]				
		2'-FL	3-FL	LNT	3'-SL	6'-SL
Infant formula as defined in Regulation (EU) No 609/2013	In the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	3.0 g/L	1.75 g/L	1.82 g/L	0.28 g/L	0.70 g/L
Follow-on formula as defined in Regulation (EU) No 609/2013	In the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	3.64 g/L	1.75 g/L	1.82 g/L	0.28 g/L	0.70 g/L
Processed cereal-based food and baby food for infants and young children as defined in Regulation (EU) No 609/2013	For beverages (liquid food ready for use) in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer	1.2 g/L	1.20 g/L	1.82 0.6 g/L	0.28 g/L	0.70 g/L
	Products other than beverages	12 g/kg	3.0 g/kg	5.0 g/kg	1.25 g/kg	2.5 g/kg
Milk-based drinks and similar products intended for young children	In the final product ready for use. marketed as such or reconstituted as instructed by the manufacturer	1.2 g/L	2.0 g/L	1.82 g/L	0.28 g/L	0.70 g/L
	Products other than beverages	-	12.0 g/kg	5.0 g/kg	-	-

Specified Food Category	Qualifiers	Maximum Use Levels[1,2]				
		2'-FL	3-FL	LNT	3'-SL	6'-SL
Unflavoured pasteurised and unflavoured sterilised (including UHT) milk-based products	-	1.2 g/L	2.0 g/L	1.0 g/L	0.25 g/L	0.5 g/L
Unflavoured fermented milk-based products	Beverages	1.2 g/L	2.0 g/L	1.0 g/L	0.25 g/L	0.5 g/L
	Products other than beverages	19.2 g/kg	5.0 g/kg	10 g/kg	0.5 g/kg	2.5 g/kg
Flavoured fermented milk-based products including heat-treated products	Beverages	1.2 g/L	2.0 g/L	1.0 g/L	0.25 g/L	0.5 g/L
	Products other than beverages	19.2 g/kg	12.0 g/kg	10 g/kg	2.5 g/kg	5 g/kg
Dairy analogues	Beverages	1.2 g/L	0.85 g/L	-	-	-
	Products other than beverages	12 g/kg	8.5 g/kg	-	-	-
	Whitener	400 g/kg	-	-	-	-
Cereal bars	-	12 g/kg	30 g/kg	10 g/kg	2.5 g/kg	5 g/kg
Table-top sweeteners	-	200 g/kg	-	-	-	-
Foods for special medical purposes as defined in Regulation (EU) No 609/2013	-	In accordance with the particular nutritional requirements of the persons for whom the products are intended				
Total diet replacement for weight control as defined in Regulation (EU) No 609/2013	Beverages	4.8 g/L	2.0 g/L	2.0 g/L	0.5 g/L	1.0 g/L
	Products other than beverages	40 g/kg for bars	30 g/kg	20 g/kg	5 g/kg	10 g/kg
Bread and pasta products bearing statements on the absence or reduced presence of gluten in accordance with the requirements of Commission Implementing Regulation (EU) No 828/2014	-	60 g/kg	-	-	-	-
Flavoured drinks	For 3'-SL and 6'-SL, uses exclude drinks with a pH less than 5.	1.2 g/L	1.25 1 g/L	1.0 g/L	0.25 g/L	0.5 g/L
Coffee, tea (excluding black tea), herbal and fruit infusions, chicory; tea, herbal and fruit infusions and chicory extracts; tea, plant, fruit and cereal preparations for infusions, as well as mixes and instant	The maximum level refers to the products ready to use	9.6 g/L	-	-	-	-

Specified Food Category	Qualifiers	Maximum Use Levels[1,2]				
		2'-FL	3-FL	LNT	3'-SL	6'-SL
mixes of these products						

[1] Taken from: *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*, as amended.

[2] In the Union List, these HMOs are also authorized for use in "food supplements". However, since the products in this category are likely not regulated as foods in Canada, the approved uses in "food supplements" are not included in this table.

D. Information on dietary intake of the nutritive substance

D.1 A detailed list of the food groups or foods in which the use of a nutritive substance is proposed, or the proposed changes to the currently permitted use levels

Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL is intended for addition alone or in combination into infant formula products that are marketed and sold in Australia.

D.2 The maximum proposed level of the use of the nutritive substance for each food group or food, or the proposed changes to the currently permitted use levels

The maximum proposed use levels of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL for addition to infant formula products are presented in Table 16 below. Note that these values are <u>not</u> inclusive of manufacturing overages.

Table 16: Maximum proposed use levels for Chr. Hansen's HMOs in Australia

Component	Maximum Proposed Level	
	Infant Formula	Follow-on Formula & SMPPi
2'-Fucosyllactose (2'-FL)	3.0 g/L	3.64 g/L
3-Fucosyllactose (3-FL)	0.9 g/L	1.2 g/L
Lacto-*N*-tetraose (LNT)	1.82 g/L	1.82 g/L
3'-Sialyllactose (3'-SL)	0.28 g/L	0.28 g/L
6'-Sialyllactose (6'-SL)	0.7 g/L	0.7 g/L

D.3 For foods or food groups not currently listed in the most recent Australian or New Zealand National Nutritional Surveys (NNSs), information on the likely level of consumption

At present, as per Schedule 29 of the Food Code, FSANZ permits the addition of the following:

- 2'-FL at a maximum level of 2.4 g/L or 96 mg/100kJ
- LNT at a maximum level of 32 mg/100kJ

- 3'-SL at a maximum level of 8mg/100kJ
- 6'-SL at a maximum level of 16 mg/100kJ

A conservative estimate of the mean and high (95th percentile) consumption levels of infant formula has been derived as **200 mL/kg bw/day** and **260 mL/kg bw/day**, respectively, by the EFSA Scientific Committee (EFSA Scientific Committee et al., 2017). The high consumption value of 260 mL/kg bw/day is considered appropriate for use in the risk assessment of substances which do not accumulate in the body and are present in foods intended for infants below 16 weeks of age (EFSA Scientific Committee et al., 2017). Similar estimations on the volume of formula consumed daily on a body weight basis have also been employed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for the risk assessment of substances for use in infant formulas (including formulas for special medical purposes intended for infants), such as pectin and octenyl succinic acid (OSA)-modified starch, as examples (Constable et al., 2017; JECFA, 2015, 2017).

The estimated daily intake (EDI) for each of Chr. Hansen's five HMOs from their maximum proposed use levels as derived based on the estimations of formula consumption volumes, is presented in Table 2. Consumption volumes of formula on a body weight basis are highest among infants at around 1 month of age and decline thereafter (Constable et al., 2017; EFSA Scientific Committee et al., 2017). Therefore, the EDI on body weight basis derived using the mean (200 mL/kg bw/day) and high (260 mL/kg bw/day) levels of formula intakes represent the most conservative scenario and are considered appropriate estimates for older infants and toddlers as well.

Even at the high consumption volume of formula of 260 mL/kg bw/day, the estimated daily intake of the five HMOs from its intended uses in formula products (*i.e.*, 780 mg/kg bw/day for 2'-FL, 195 mg/kg bw/day for 3-FL, 390 mg/kg bw/day for LNT, 60 mg/kg bw/day for 3'-SL, and 73 mg/kg bw/day for 6'-SL) are comparable to those of infants fed human milk, as described above in Section 3.1. Although the EDI slightly exceeds the "high" intake levels estimated for 2'-FL (767 mg/kg bw/day) and LNT (287 mg/kg bw/day) by breastfed infants, it should be recognised that:

- Wide variability exists in the concentrations of HMOs in human milk. The upper levels of intake for HMOs by breastfed infants were calculated using "maximum mean" concentrations for the HMOs in mature human milk (reflecting Day 15 to 90 of lactation), as reported in the review by Soyyilmaz et al. (2021). Levels that are even higher than the "maximum mean" concentration for LNT was derived as 1.6 g/L for mature breastmilk, it was reported at 3.9 g/L for transitional milk (reflecting Day 6 to 14 of lactation) (Soyyilmaz et al., 2021). Moreover, in EFSA's safety evaluation of LNT as a novel food, a "high" concentration level of 2.74 g/L was selected for the calculation of the potential exposure to LNT by infants fed human milk (EFSA NDA Panel, 2019b).
- The 95th percentile intake of infant formula consumption used in the derivation of the EDIs (260 mL/kg bw/day) is a highly conservative estimate.

These caveats of the exposure assessment have also been recognised by other authoritative bodies in their safety evaluation of HMOs as formula ingredients. As an example, in a recent opinion on the safety of 3-FL as a novel food published by the EFSA NDA Panel (EFSA NDA Panel, 2021), which included uses in formula products and other foods for infants and young children, it was concluded that:

"The Panel also notes that the anticipated daily intake of 3-FL in the NF [novel food] *from the consumption of IF* [infant formula] *only, in infants up to 16 weeks of age, does not exceed the highest intake level of 3-FL in breastfed infants on a body weight basis. In consideration of the wide variability observed in human milk levels and the conservative assumption underlying the estimated intake, the exceedance at high (95th percentile) intake noted in infants below 1 year of age (in only one out of 13 dietary surveys included in the EFSA food consumption database) does not raise safety concerns."*

Refer to Table 2 for the estimated daily intake for HMOs.

D.4 The percentage of the food group to which the use of the nutritive substance is proposed or the percentage of the market likely to use the nutritive substance

In estimating the intake of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL (alone or in combination) from their proposed use in infant formula products, it can be assumed that these HMOs will be added to formula products in these aforementioned categories.

However, it is unlikely that the inclusion of Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL (used alone or in combination) will have 100% market penetration. Realistically, the application of this assumption in the exposure estimate will provide a conservative estimate of the consumption of these five HMOs under the proposed conditions of use.

Additionally, it can be noted that this application seeks exclusive permission for Chr. Hansen to market its 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL ingredients as foods produced using gene technology (see **Section 3.1.1 I**)

D.5 Information relating to the use of the nutritive substance in other countries

Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL can be found used individually and in combination in infant formula and follow-on formula globally in Canada, India, the United States of America, Europe, Singapore, Malaysia, China, Saudi Arabia, Israel, the Philippines and others.

D.6 For foods where consumption has changed in recent years, information on likely currently food consumption

Not applicable.

F. Information related to the nutritional impact of a nutritive substance other than vitamins and minerals

F.1 Information related to the nutritional purpose of the use of the substance in each food

Chr. Hansen's 5HMO-Mix is intended for addition into infant formula products that are marketed in Australia. The levels of the five individual HMO components of 2'-FL, 3-FL, LNT, 3'-SL, 6'-SL have been described in **Section 3.3.3 D.2** above. These inclusion rates were selected to reflect the concentrations that have been reported for the corresponding HMOs naturally present in human milk.

G. Information related to potential impact on consumer understanding and behaviour

G.1 Information to demonstrate the level of consumer awareness and understanding of the nutritive substances in the food(s)

Based on publicly available information and information from Australian health advisories and breastfeeding associations, it can be expected that the majority of parents and caregivers are aware of the positive health benefits of breastfeeding.

Historically in Australia, inulin-type fructans have been permitted for addition to infant formula products, infant foods, and formulated supplementary foods for young children (FSFYC) whether alone or in combination with galacto-oligosaccharides. The purpose of adding these oligosaccharides to infant and follow-on formula is to mimic the effects of these oligosaccharides as they occur naturally in breast milk (FSANZ, 2008).

At present, there is no direct data on consumer awareness and understanding of HMOs from peer-reviewed scientific journals. However, it can be expected that consumers are aware and capable of understanding the benefits resulting from consumption of formula products that contain ingredients to enable a closer comparison to human milk especially for mothers/ caregivers who are unable to or choose not to breastfeed.

G.2 Information on the actual or potential behaviour of consumers in response to proposed food(s)

Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL, used alone and in combination, have already been commercialised into infant formula products in several countries, including but not limited to the EU, US, and others. The anticipated response of consumers in Australia to the entry of the abovementioned HMOs is expected to be comparable to that which has already been seen globally for similar non-digestible oligosaccharides.

The recommendation from Australia's National Health and Medical Research Council (NHMRC) is to exclusively breastfeed for approximately the first 6 months, and for breastfeeding to continue alongside complementary foods for 1 year or as long as the mother and child desire. At present, infant formula is the only suitable and safe alternative to breastfeeding capable of meeting an infant's primary nutritional needs.

The purpose of adding 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL to infant formula products is to make its composition closer to that of human breast milk, which is the gold-standard for infant feeding. As such, it is expected that such products will be well-received by mothers and caregivers who are unable to or choose not to breastfeed.

G.3 Information to demonstrate that the consumption of food(s) containing the nutritive substance will not adversely affect any population groups (e.g., age, or cultural groups)

All infant formula products sold in Australia are regulated by the Australia New Zealand Food Standards Code and contain adequate nutrients for infants. Additionally, the quality, composition, and labelling of infant formula products are regulated under Standard 2.9.1. The estimated daily intakes for 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL have been described in **Section 3.3.3 D.3** of this application.

As Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL are chemically equivalent to those found in human milk, products containing Chr. Hansen's HMOs are able to better imitate human milk than those products not containing these HMOs. Therefore, products containing Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL alone or in combination are not expected to adversely affect any population groups in Australia.

3.5.1 Foods Produced by Gene Technology

A. Technical information on the food produced using gene technology

A.1 Nature and identity of the genetically modified food

Description of the GM organism from which the new GM food is derived

Taxonomic classification of the host strain

The HMOs *Escherichia coli* BL21(DE3) production strains are based on the host organism *Escherichia coli* BL21(DE3), and its taxonomic classification is shown in the table below.

Table 17: Scientific classification for *Escherichia coli* BL21(DE3)

Domain	Bacteria
Kingdom	Eubacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	Escherichia
Species	*Escherichia coli*
Strain	*Escherichia coli* BL21(DE3)

The *E. coli* B strain was originally used to study the function of T-phage, so in 1940 it was widely distributed among laboratories all over the world (such as Delbrück, Luria and Hershey laboratories). *E. coli* (especially B strain) is a mature microorganism used for commercial production of therapeutic proteins. The first biopharmaceutical produced by the fermentation of genetically modified cells (recombinant insulin; approved in 1982, marketed as Humulin) was the *E. coli* B strain.

Since the strain was isolated in 1918, the *E. coli* B strain has been genetically manipulated several times and hence created the strain BL21(DE3).

In the 1960s, the derivation of *E. coli* BL21(DE3) strain has been fully documented in the literature (Daegelen, Studier, Lenski, Cure, & Kim, 2009). The early derivatives of *E. coli* used ultraviolet light for mutations and auxotrophic introduction of gene fragments with methionine, but the biggest genetic modification was to insert the bacteriophage T7 RNA polymerase gene in the gene body to derive the BL21(DE3) strain (Studier & Moffatt, 1986). *E. coli* BL21(DE3) is probably the most widely used bacterial strain that overexpresses exogenous and endogenous recombinant proteins.

Identity of the GM organism from which the food is derived

The OECD unique identifiers are assigned to transgenic plants. *E. coli* B strain is a bacterium found in the mammalian colon and widely used for metabolic engineering. Thus, *E. coli* BL21(DE3), the GM

organism used as a processing aid in the manufacture of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL, has neither a line number nor an OECD unique identifier.

Name the food will be marketed under (if known)

Not available.

A.2 History of use of the host and donor organisms

E. coli BL21 (DE3) is classified as a risk group 1 organism, the lowest possible risk group according to the German Federal Office of Consumer Protection and Food Safety (BVL).

Escherichia coli (and particularly the *E. coli* B strain) is a well-established microorganism for the commercial production of therapeutic proteins. The first biopharmaceutical product produced in genetically modified cells was manufactured by the fermentation of an engineered *E. coli* B strain (recombinant insulin, marketed as Humulin, approved in 1982). *E. coli* BL21 (DE3) is probably the most widely used bacterial strain for the overexpression of heterologous and homologous recombinant proteins.

The *E. coli* B strain was widely distributed among laboratories throughout the world during the 1940s, particularly due to the initial use of *E. coli* B to study T-phage functions (e.g., in the laboratories of Delbrück, Luria and Hershey). Since its isolation in 1818, the *E. coli* B strain has also undergone multiple rounds of genetic manipulation resulting in the strain BL21 (DE3). The derivation of *E. coli* BL21 (DE3) is well documented since the 1960s (Daegelen et al., 2009). Early modifications of *E. coli* B related to UV mutagenesis and the introduction of genetic elements for methionine auxotrophy, but the most significant genetic modification was the genomic integration of the bacteriophage T7 RNA polymerase gene, which resulted in the generation of strain BL21 (DE3) (Studier & Moffatt, 1986).

A.3 The nature of the genetic modification

Each of the production strains employed in the manufacture of Chr. Hansen's five HMOs are derivatives of *E. coli* BL21(DE3). A series of common genetic modifications are introduced into the *E. coli* BL21(DE3) parental strain, which is then further engineered to allow for the biosynthesis of the specific HMO of interest. A tabular listing of the genetic modifications in the strain, which are present in all of the production strains used to manufacture Chr. Hansen's five HMOs, is presented in Appendix 08 (**COMMERCIAL CONFIDENTIAL INFORMATION**). The additional modifications that are further introduced to allow for the production of the individual HMOs, namely 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL, are listed in Appendix 08 (**COMMERCIAL CONFIDENTIAL INFORMATION**). An additional "degradation strain" may be optionally employed for the production process of LNT, 3'-SL, and 6'-SL to degrade excess lactose and carbohydrate by-products remaining in the fermentation medium. The modifications in these optional degradation strains are also listed in Appendix 08 (**COMMERCIALLY CONFIDENTIAL INFORMATION**).

All genes integrated into individual production strains and optional degradation strains are well-characterized. Additionally, all plasmids and/or episomal vectors were removed during the engineering process. Full details of the construction and characterization of the genetically modified *E. coli* BL21(DE3) production strains are presented in Appendix 08 (**COMMERCIAL CONFIDENTIAL INFORMATION**).

All strains are stored at the production site as glycerol stocks in a master cell bank at -80°C. The glycerol stocks are used to produce working cell banks, which are then used for the production of the individual HMOs. All strains have been deposited at the DSMZ (Deutsche Sammlung für Mikroorganismen und Zellkulturen) German Collection of Microorganisms and Cell Cultures.

The genetic stability of each *E. coli* BL21(DE3) HMO production strain was evaluated through a structured study covering five consecutive culture generations. The assessment included genomic stability (PCR analysis of all integrated genes and gene clusters), protein expression stability (targeted proteomics of key enzymes encoded by the inserted operons), and product stability (HMO titers determined by LC–MS/MS using multiple reaction monitoring). PCR analyses confirmed the integrity of all introduced genetic elements, with amplicons of the expected size detected in all clones across all generations, demonstrating the absence of deletions or rearrangements. Targeted proteomic analyses confirmed consistent expression of all strain-specific proteins in each generation, with no evidence of loss or reduction of protein abundance. In addition, mass spectrometry demonstrated stable production of the respective HMOs across all generations, with no observed decrease in titers. Taken together, these results confirm that each HMO production strain is genetically stable. The strains consistently maintain the integrity of integrated genes, express the relevant biosynthetic enzymes, and produce HMOs at stable levels. Consequently, robust and reproducible fermentation processes are assured, ensuring consistent product quality. Stability reports for the inserted genes are provided in Appendix 23 (**COMMERCIAL CONFIDENTIAL INFORMATION**) including details of the test methods using PCR, proteomic analysis using LC/MS, and HMO titer as summarized above. Data on the genetic stability has been previously provided on 2'-FL as part of Application A1190.

Additionally, the details of modifications to the DNA and amino acid sequences for the heterologous genes inserted into the production strains are provided in Appendix 24 (**COMMERCIAL CONFIDENTIAL INFORMATION**). Data on the modifications to the DNA an amino acid sequences has been previously provided on 2'-FL as part of Application A1190.

B. Characterisation and safety assessment of new substances

B.1 Characterisation and safety assessment of new substances

The phenotypic traits and biochemical characterisation of *E. coli* (BL21) DE3 are detailed in **Section 3.5.1 A.3** and Appendix 08 (**COMMERCIAL CONFIDENTIAL INFORMATION**). The *E. coli* BL21(DE3) strains are genetically engineered to export 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL respectively into the fermentation medium during the HMO manufacturing process (details in **Section 3.3.3 B.4**). The new substances are chemically and structurally identical to their naturally occurring equivalents found in human milk (see **Section 3.3.3 B.2.2**) and has the same nutritional and immunity potential in infants. Furthermore, 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL have a history of safe consumption by infants (see **Section 3.3.3 A.1**).

B.3 Other (non-protein) new substances

The biological function of the HMOs in this application is explained in **Section 3.3.3 A.1** of this application. The structural and chemical similarities between Chr. Hansen HMOs and naturally occurring HMOs are detailed in **Section 3.3.3 B.2.2**. The potential dietary exposure to these HMOs is described in **Section 3.3.3 A.1** and **Section 3.3.3 D.2**. **Section 3.3.3 D.5** of this application provides a list of countries where products containing Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL have been launched.

B.5 Compositional analyses of the food produced using gene technology

The addition of Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL and 6'-SL to infant formula products, is consistent with levels similar to those that are naturally occurring in human milk. The structural and chemical similarities between Chr. Hansen's HMOs and naturally occurring HMOs are detailed in **Section 3.3.3 B.2.2** and Appendix 07 (**COMMERCIAL CONFIDENTIAL INFORMATION**). Chr. Hansen's HMOs are substantially chemically equivalent to naturally occurring HMOs.

As per the previously described manufacturing method used, unintended effects due to the genetic modification of the processing aid are highly unlikely (see **Section 3.3.3 B.4**).

The proposed use of Chr. Hansen's HMOs is described in **Section 3.3.3 D.2**.

C. Information related to the nutritional impact of the food produced using gene technology

Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are substantially chemically equivalent to naturally occurring 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL in human milk (see **Section 3.3.3 B.2.2** and Appendix 07 (**COMMERCIAL CONFIDENTIAL INFORMATION**)). No biologically significant changes are expected in these HMOs when produced by microbial fermentation as compared to naturally occurring in human milk.

Clinical studies carried out with infant formula supplemented with these HMOs alone and in combination show that 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL are well-tolerated (see **Section 3.3.3 C.2.4** and **C.2.5**). Additionally similar ingredients have been assessed by FSANZ and have been approved for use in Australia. These applications have been listed in Table 12 above.

As such, no nutritional impact is expected from the use of 2'FL, 3-FL, LNT, 3'-SL, and 6'-SL.

D. Other Information

Refer to **Section 3.3.3 C.2.1-3** for details on toxicity studies conducted on animals.

3.6.2 Special Purpose Food – Infant Formula Products

A. Information related to composition

A.1 Purpose of the compositional change

Information on the purpose of adding 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL to infant formula products individually and in combination has been described in **Section 3.3.3 A.1** of this application.

A.2 General data requirements

Additional supporting evidence for the addition of 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL to infant formula products are described in the following sections of this application:

- The history of safe consumption of these five HMOs (individually and in combination) as well as their beneficial properties is described in **Section 3.3.3 A.1**.
- The results of toxicology studies are described in **Sections 3.3.3 C.2.1-3**.
- The results of studies carried out using Chr. Hansen's 5HMO-Mix are presented in **Section 3.3.3 C.2.4**, while the results of clinical studies carried out using individual HMOs are described in **Section 3.3.3 C.2.5**.

A.3 Specific information requirements for the nutritional safety, tolerance, and efficacy of the proposed compositional change

A.3.1 Nutritive substance, novel food, or novel food ingredient

Information on the safety, tolerance, and efficacy of Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL have been described in the following sections of this application:

- The beneficial properties of these five HMOs is described in **Section 3.3.3 A.1**.
- The safety and tolerance of these five HMOs is described in **Section 3.3.3 C**.
- The concentrations of these five HMOs in human breast milk is described in Appendix 05.

B. Information related to the dietary intake or dietary exposure

B.1 Data to enable the dietary intake or exposure of the target population to be estimated

Refer to **Section 3.3.3 A.1** and **Section 3.3.3 D.2** for information related to dietary intake and dietary exposure of Chr. Hansen's 5HMO-Mix.

B.2 Data on the recommended level of formula consumption

Chr. Hansen's HMOs are intended to be ingredients in infant formula products in Australia at levels as described in **Section 3.3.3 D.2**. These proposed levels are comparable to the levels of these HMOs are present in human milk.

As such, there are no proposed changes to the formula preparation instructions regarding the capacity of the product scoop, number of scoops required per feed, volume of water required per feed, or total volume of the made-up feed. The consumers will be required to follow the preparation instructions as provided by the manufacturer.

B.3 Information relating to the substance

Refer to **Section 3.3.3 A.1** of this application for information relating to the presence of 2'-FL, 3-FL, LNT, 3'-sL, and 6'-SL in human milk. The inclusion of synthesised 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL is not currently permitted by FSANZ in infant food products such as infant cereals. Therefore, it is not anticipated that Australian infants will be exposed to 2'-FL, 3-FL, LNT, 3'-SL, and/or 6'-SL in other foods that they are likely to consume except for breast milk in cases where complimentary feeding is occurring.

C. Information related to labelling requirements under Part 2.9 of the Code

C.1 Information related to safety or nutritional impact of the proposed labelling change

There is no expected change to the infant formula product labelling requirements due to the addition of Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL alone or in combination to formula sold in Australia.

C.2 Information to demonstrate that the proposed labelling change will be understood and will assist consumers

There is no expected change to the infant formula product labelling requirements due to the addition of Chr. Hansen's 2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL alone or in combination to formula sold in Australia. Chr. Hansen's HMOs meets the exception listed under Standard 1.5.2-4(a) in that Chr. Hansen's HMOs "has been highly refined where the effect of the refining process is to remove novel DNA or novel protein" and is not listed in subsections S26-3(2) and (3).

D. Information related to internationally recognised standards, codes, or practice, recommendations, AND guidelines

Section 3.1.1 D and **Section 3.1.1 J.1** of this application provides information relating to internationally recognised standards and recommendations for infant formula that recommend a composition as close to the natural composition of breast milk.

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