Application to Amend the Australia New Zealand Food Standards Code to Use 3-Fucosyllactose Produced using Gene Technology as a Nutritive Substance in Infant Formula Products

19 December 2024

Submitted by:
Glycom A/S
Kogle Allé 4
2970 Hørsholm
Denmark

Glycom A/S is ultimately controlled by DSM-Firmenich AG, registered in Kaiseraugst, Switzerland.

Application to Amend the Australia New Zealand Food Standards Code to Use 3-Fucosyllactose Produced using Gene Technology as a Nutritive Substance in Infant Formula Products

Contents

List of Tables

List of Figures

Appendices

Application to Amend the Australia New Zealand Food Standards Code to Use 3-Fucosyllactose Produced using Gene Technology as a Nutritive Substance in Infant Formula Products

Introduction

Glycom A/S (Glycom herein), ultimately controlled by DSM-Firmenich AG (registered in Kaiseraugst, Switzerland), is seeking to amend Schedules 3, 26 and 29 of the Australia New Zealand Food Standards Code (the Code) to permit the use of 3-Fucosyllactose (3-FL) produced by microbial fermentation as a nutritive substance in infant formula products in Australia and New Zealand.

3-FL is a human milk oligosaccharide (HMO). The manufactured 3-FL has identical structure to 3-FL naturally present in human milk. It is a trisaccharide consisting of D-glucose, D-galactose, and L-fucose, and is a simple structural isomer of 2′-fucosyllactose (2′-FL) already permitted for use as a nutritive substance in infant formula products in Australia and New Zealand. A unique characteristic of 3-FL is that, unlike most other HMOs, its concentration increases in breastmilk as lactation progresses. Furthermore, 3-FL is present in the breastmilk of all women, irrespective of their Secretor status.

The manufactured 3-FL is produced by microbial fermentation using an *Escherichia coli* (*E. coli*) K-12-derived production strain, similar to other HMOs manufactured by Glycom already permitted for use as nutritive substances in infant formula products in Australia and New Zealand: 2′-Fucosyllactose (2′-FL), lacto-*N*-neotetraose (LNnT), a combination of 2′-fucosyllactose and difucosyllactose (2′-FL/DFL), lacto-*N*-tetraose (LNT), 3′-sialyllactose sodium salt (3′-SL), and 6′-sialyllactose sodium salt (6′-SL) (Schedule 26). The 3-FL production strain has been modified to contain the gene necessary for the biosynthesis of 3-FL, namely alpha-1,3-fucosyltransferase. The donor gene from *Helicobacter pylori* was not isolated or directly amplified from the donor organism but rather derived from *de novo* DNA synthesis based on defined DNA sequences obtained from bioinformatic databases. During fermentation, 3-FL is secreted extracellularly from the fermentation organism into the culture medium. The fermentation organism is removed, and the 3-FL is isolated and concentrated by a series of steps, resulting in a highly purified 3-FL ingredient.

Glycom's 3-FL manufactured by microbial fermentation has gained novel food approval in the European Union (EU) and the United Kingdom (UK), and it has achieved Generally Recognized as Safe (GRAS) status in the United States of America (USA). It is also included on the Therapeutics Good (Permissible Ingredients) Determination for use in complementary medicines in Australia.

3-FL is intended to be used in Australia and New Zealand as a nutritive substance in infant formula products, alone or in combination with other permitted HMOs, at levels within the range of human breastmilk. The maximum proposed use level of 2.0 g/L has already been evaluated and determined to be safe by the UK's Advisory Committee on Novel Foods and Processes (ACNFP).

This dossier has been prepared for evaluation in accordance with the following Guidelines in the Food Standards Australia New Zealand (FSANZ) Application Handbook[1]:

- Guideline 3.1.1: General requirements
- Guideline 3.3.3: Substances used for a nutritive purpose
- Guideline 3.5.1: Foods produced using gene technology
- Guideline 3.6.2: Special purpose foods (infant formula products)

Information to demonstrate that Glycom's 3-FL is safe and appropriate under the intended conditions of use are detailed herein.

[1] Application Handbook | Food Standards Australia New Zealand

Part 3.1.1 – General Requirements

B. Applicant Details

Glycom A/S is the applicant. Glycom Manufacturing A/S is the manufacturing site. Both Business Entities are ultimately controlled by DSM-Firmenich AG, registered in Kaiseraugst, Switzerland. They are dedicated to the scientific and commercial development of human milk oligosaccharide ingredients.

Applicant:	**Glycom A/S** Kogle Allé 4 2970 Hørsholm Denmark Tel +45 88 30 95 20
Manufacturing site:	**Glycom Manufacturing A/S** Limfjordsvej 4 6715 Esbjerg Denmark Tel +45 88 30 95 00
[redacted]	[redacted]

C. Purpose of the Application

Human milk contains a number of structurally diverse oligosaccharides, termed human milk oligosaccharide (HMOs). Glycom has developed methods to manufacture some of the most abundant HMOs by fermentation with *E. coli* K-12 derivative strains containing biosynthetic genes for their production. HMOs produced by microbial fermentation are chemically and structurally identical to the same oligosaccharides that are naturally present in human milk.

Several HMOs produced using gene technology of microbial origin are already permitted for use in infant formula products in Australia and New Zealand (Schedule 26), including six of Glycom's HMOs produced by *E. coli* K-12 derivative strains, summarised in Table 1.

Table 1 HMOs Manufactured by Glycom Already Permitted for Use in Australia and New Zealand

Application	Substance	Source
A1155	2′-Fucosyllactose (2′-FL)	*Escherichia coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori*
	Lacto-N-neotetraose (LNnT)	*Escherichia coli* K-12 containing the gene for beta-1,3-N-acetylglucosaminyltransferase from *Neisseria meningitides* and the gene for beta-1,4-galactosyltransferase from *Helicobacter pylori*
A1265	A combination of 2′-fucosyllactose and difucosyllactose (2′-FL/DFL)	*Escherichia coli* K-12 containing the gene for alpha-1,2-fucosyltransferase from *Helicobacter pylori*
	Lacto-N-tetraose (LNT)	*Escherichia coli* K-12 containing the gene for beta-1,3- N-acetylglucosaminyltransferase from *Neisseria meningitides* and the gene for beta-1,3- galactosyltransferase from *Helicobacter pylori*
	6′-sialyllactose sodium salt (6′-SL)	*Escherichia coli* K-12 containing the gene for alpha-2,6-sialyltransferase from *Photobacterium damsela* and CMP-Neu5Ac synthetase, Neu5Ac synthase, N-acetylglucosamine-6-phosphatase epimerase from *Campylobacter jejuni*
	3′-sialyllactose sodium salt (3′-SL)	*Escherichia coli* K-12 containing the gene for alpha-2,3-sialyltransferase from *Neisseria meningitides* and CMP-Neu5Ac synthetase, Neu5Ac synthase, N-acetylglucosamine-6-phosphatase epimerase from *Campylobacter jejuni*

HMOs are intended to be added to infant formula products to replicate the abundance of these naturally occuring oligosaccharides in human milk and distinct biological functions associated with their diverse structures. Accordingly, infant clinical studies have been conducted evaluating the safety and benefit of the addition of various combinations of HMOs to infant formula compared to infant formula without HMOs (see Part 3.3.3, Sections A.2.2 and C.2.2), and infant formula products containing up to 6 HMOs (including 3-FL) have been commercialised globally (see Part 3.3.3, Section G.3).

3-FL is among the 10 most abundant HMOs in human breastmilk, and unlike most other HMOs, its concentration in human milk increases as lactation progresses (Soyyilmaz *et al.*, 2021).

The purpose of this application is to amend Schedule 26 to include 3-FL as a food produced using gene technology of microbial origin for use in infant formula products. It is recognised

that the approval of 3-FL under the proposed amendment will require consideration and possible changes to the following Standards and their associated Schedules, as relevant:

- Standard 2.9.1: Infant formula products;
- Schedule 3: Identity and purity;
- Schedule 26: Food produced using gene technology (of microbial origin); and
- Schedule 29: Special purposed foods.

Information and data presented in this application support the safe and suitable use of 3-FL for its proposed use in infant formula products.

D. Justification for the Application

D.1 Regulatory Impact Information

D.1.1 Benefits of the Application

a) Consumer Benefits

Human milk contains more than 200 structurally diverse oligosaccharides, termed human milk oligosaccharides (HMOs) (Kunz *et al.*, 2000; EFSA, 2014; Soyyilmaz *et al.*, 2021). HMOs are the third most abundant solid component of human milk (Hester *et al.*, 2012), reaching concentrations of up 25 g/L in human colostrum and up to 20 g/L in mature human milk (Bode, 2012).

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

Glycom has developed the technology to manufacture a number of HMOs by microbial fermentation that are structurally and chemically identical to their counterparts that are

naturally present in human milk. 3-FL is among the most abundant HMOs that constitute the majority of the total HMO composition in mature human milk (Soyyilmaz *et al*., 2021). Furthermore, the concentration of 3-FL is higher in mature milk than colostrum, contrary to other HMOs that decrease or stabilise in concentration after colostrum (Soyyilmaz *et al*., 2021).

3-FL manufactured by Glycom is intended for addition to infant formula products alone or in combination with other manufactured HMOs permitted for use in Australia and New Zealand. The maximum proposed use level of 3-FL for addition to infant formula products of 2.0 g/L (equivalent to 80 mg/100 KJ) is consistent with mean concentrations of 3-FL in mature human milk reported in the literature (see Part 3.6.2, Section A.3.1.2).

The safety of the addition of 3-FL in combination with other HMOs to infant formula products has been evaluated in three randomised controlled trials conducted in healthy term infants (Clinical Trial Registry: NCT03513744, NCT04105686, and NCT04962594). In all three clinical trials, the experimental formulas containing 3-FL supported age-appropriate growth and were safe and well-tolerated (see Part 3.3.3, Section C.2.2).

The benefit of the addition of 3-FL in combination with other HMOs to infant formula products has been evaluated in two of the three clinical trials (Clinical Trial Registry: NCT03513744 and NCT04962594). In both clinical trials, the experimental formulas containing 3-FL shifted the composition of the gut microbiome closer to that of breastfed infants by increasing the abundance of beneficial *Bifidobacteria* and decreasing pathogenic bacteria (see Part 3.3.3, Section A.2.2).

Increased abundance of beneficial *Bifidobacterium spp.* in the infant gut microbiome and anti-pathogenic effects have previously been considered by FSANZ as beneficial health effects associated with the addition of HMOs to infant formula (FSANZ, 2019, 2020, 2023a).

b) Industry Benefits

3-FL is already authorised for use in infant formula products in other markets (including EU, UK, and USA). Infant formula products with different combinations of HMOs (including 3-FL) have been evaluated clinically and are commercially available. Therefore, 3-FL approval in Australia and New Zealand would increase accessibility to a greater variety of infant formula products for importation. It also offers increased choice of ingredients to industry for innovation and improvement of infant formula products, as well as opportunity to tailor HMO combinations according to commercial benefit for infant formula products sold domestically or exported to international markets.

c) Government Benefits

As mentioned above, 3-FL is already authorised for use in infant formula products in other jurisdictions (EU, UK and USA). Therefore, approval of 3-FL would further harmonise HMO approvals between Australia and New Zealand and these regions.

D.1.2 Impact on International Trade

Approval of 3-FL for use in infant formula products would increase trade opportunities (import and export) between Australia and New Zealand and other overseas countries that already have 3-FL authorisation (EU, UK and USA).

E. Information to Support the Application

This application is prepared in accordance with the relevant sections in the Food Standards Australia New Zealand Application Handbook (from 01 July 2024):

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

Comprehensive literature searches have previously been conducted as part of regulatory submissions for 3-FL in the EU, UK and USA. Glycom's 3-FL is permitted for use in a variety of foods (including infant formula products) in these jurisdictions following safety assessment by regulatory authorities, including the European Food Safety Authority (EFSA, 2023), the UK ACNFP (ACNFP, 2024), and the United States Food & Drug Administration (U.S. FDA, 2022). The literature has been monitored for new, relevant, publications reporting on the safety and benefit of 3-FL, which are included in this application.

F. Assessment Procedure

Glycom considers the **General Procedure (Level 3)** to be the most appropriate assessment procedure for the evaluation of this application to amend the Code to permit the use of 3-FL produced using gene technology in infant formula products.

Six other HMO products manufactured by Glycom using the same *E. coli* K-12-derived platform strain for HMO production have already been assessed by FSANZ and are authorised for use in the same food category [2'-FL and LNnT (Application A1155); 2'-FL/DFL, LNT, 3'-SL sodium salt and 6'-SL sodium salt (Application A1265)].

The safety and benefit of the proposed use of 3-FL is largely based on the history of consumption of this HMO by breastfed infants from human milk and is supported by pre-clinical and clinical data.

3-FL has been previously assessed by other regulatory authorities and determined to be safe (EFSA, UK ACNFP, and U.S. FDA), and is authorised for use and commercially available in several markets (EU, UK, and USA).

G. Confidential Commercial Information (CCI)

Confidential commercial information, in relation to food, is defined in Section 4 of the FSANZ Act[2] as:

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

Glycom requests the information contained within the following Appendices be considered confidential commercial information (CCI):

- Appendix 3 – NMR Spectral Analysis
- Appendix 4 – Stability Studies
- Appendix 5 – Certificates of Analysis & Batch Data
- Appendix 6 – Manufacturing Process
- Appendix 7 – Internal Methods of Analysis
- Appendix 8 – Unpublished Study Reports
- Appendix 9 – Post-Market Surveillance Data
- Appendix 10 – Production Strain Data

The information contained within these appendices is not publicly available and release of these data would be at a commercial disadvantage to Glycom, having invested considerable capital to establish the manufacturing process of a high purity 3-FL by microbial fermentation, develop and validate methods of analyses, conduct testing to confirm the quality and stability of the ingredient, and commission ingredient-specific toxicological studies to confirm safety.

[2] Federal Register of Legislation - Food Standards Australia New Zealand Act 1991

H. Other Confidential Information

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

I. Exclusive Capturable Commercial Benefit (ECCB)

According to Section 8 of the FSANZ Act[3], an exclusive capturable commercial benefit is conferred on an applicant if:

a) the applicant can be identified as a person or body that may derive a financial gain from the coming into effect of the draft standard or draft variation of the standard that would be prepared in relation to the application; and
b) any other unrelated persons or bodies, including unrelated commercial entities, would require the agreement of the applicant in order to benefit financially from the approval of the application.

The application is expected to confer an exclusive capturable commercial benefit to Glycom, as 3-FL is a novel food ingredient produced *via* a proprietary manufacturing process that has been pre-clinically and clinically tested and is commercially available in overseas markets. There has been significant research and investment by Glycom and its partners into the development of this HMO ingredient, to increase access to manufactured HMOs for addition to infant formula products to more closely reflect the natural composition of human milk, and for formula-fed infants to benefit from associated health outcomes.

It is envisioned that exclusivity will be specific to 3-FL sold under the GlyCare® brand for addition to infant formula products, and not to finished food products containing the material. In practice, this means that during the exclusivity period, a manufacturer may only incorporate 3-FL from the GlyCare® brand into their infant formula products, under the authorised conditions of use.

J. International and Other National Standards

J.1 International Standards

Codex Alimentarius (Codex) International Food Standards do not currently exist for HMOs (including 3-FL). Nevertheless, the Codex Standards for 'Infant Formula and Formulas for Special Medical Purposes Intended for Infants' (Codex Alimentarius, 2023a) and for 'Follow-Up

[3] Federal Register of Legislation - Food Standards Australia New Zealand Act 1991

Formula for Older Infants and Product for Young Children' (Codex Alimentarius, 2023b) contain provisions for 'optional ingredients' that are applicable to the intended use of 3-FL in infant formula products, summarised in Table 2.

Table 2 Codex Alimentarius Provisions Applicable to the Intended Use of 3-FL in Infant Formula Products

Standard	Provisions
Infant formula and formulas for special medical purposes intended for infants (CXS 72-1981)	• Optional ingredients may be added in order to provide substances ordinarily found in human milk and to ensure that the formulation is suitable as the sole source of nutrition for the infant or to provide other benefits that are similar to outcomes of populations of breastfed babies. • The suitability for the particular nutritional uses for infants and the safety of these substances shall be scientifically demonstrated. The formula shall contain sufficient amounts of these substances to achieve the intended effect, taking into account levels in human milk.
Follow-up formula for older infants (CXS 156-1987)	• Optional ingredients may be added where the safety and suitability of the optional ingredient for particular nutritional purposes, at the level of use, is evaluated and demonstrated by generally accepted scientific evidence. • When any of these ingredients is added, the formula shall contain sufficient amounts to achieve the intended effect, taking into account levels in human milk.

3-FL = 3-fucosyllactose.

J.2 Other National Standards and Regulations

National standards for 3-FL in jurisdictions with comparable regulatory processes that are relevant to the current application are summarised in Table 3.

Table 3 3-FL Approvals in Other Jurisdictions

Jurisdiction	National Standard
EU	Glycom's 3-FL produced by derivative strain of *E. coli* K-12 DH1 is authorised for use as novel food ingredient under Commission Implementing Regulation (EU) 2023/2210 (EU, 2023), for which provisions are laid down in the current consolidated version of the Union list of novel foods[4].
UK	Glycom's 3-FL produced by a derivative strain of *E. coli* K-12 DH1 is authorised for use as novel food ingredient *via* Statutory Instruments from England (UK Government, 2024a), Wales (UK Government, 2024b), and Scotland (UK Government, 2024c).

[4] Implementing regulation - 2017/2470 - EN - EUR-Lex

Jurisdiction	National Standard
USA	Glycom's 3-FL produced by microbial fermentation have been notified as GRAS to the U.S. FDA (GRN 1037[5]), and has received a no questions letter from the Agency (U.S. FDA, 2022).

3-FL = 3-fucosyllactose; EU = European Union; GRAS = generally recognized as safe; UK= United Kingdom; USA = United States of America; U.S. FDA = United States Food & Drug Administration.

K. Statutory Declaration

A signed Statutory Declaration is provided in Appendix 1 **(Confidential)**.

L. Checklists

Completed checklists relating to the information required for submission with this application are provided in Appendix 2.

[5] GRN 1037 - Glycom A/S - 3-FL

Part 3.3.3 – Substances Used for a Nutritive Purpose

A. Information on the Use of the Nutritive Substance

A.1 Purpose of the Use of the Substance

3-FL is an oligosaccharide that naturally occurs in human milk. Collectively, HMOs represent the third most abundant solid component of breastmilk (Urashima *et al*., 2012), and more than 200 HMO structures have been identified (Ninonuevo *et al*., 2006). According to EFSA's most recent updated Scientific Opinion on the essential composition of infant and follow-on formula, HMOs are principal growth factors for bifidobacteria in the infant gut and influence the composition of the gut microbiota in breastfed infants (EFSA, 2014). In addition to the well-established role of HMOs in shaping the infant gut microbiota, these non-digestible oligosaccharides have also been demonstrated to have anti-pathogenic effects, maintain intestinal barrier integrity, have immunomodulatory effects, and influence cognitive development, as previously summarised in Applications A1155 and A1265.

Infant formula products are considered to be the only suitable breastmilk substitutes for infants (Codex Alimentarius, 2023a,b). Currently, the majority of infant formula and follow-on formula products on the market are formulated with mature cow's milk. Oligosaccharides are present in other mammalian milks, but not to any significant degree compared to human milk (see Part 3.6.2, Section B.3). As such, the absence of human milk oligosaccharides in infant formula products remains the biggest compositional difference compared to human milk.

3-FL is a fucosylated HMO and is among the 10 most abundant oligosaccharides in human milk (Soyyılmaz *et al*., 2021). Unlike 2'-FL, secretor status does not impact the occurrence of 3-FL in breastmilk (as α-1,3-fucosyl-transferase is encoded by several enzymes), and the concentration of 3-FL increases throughout lactation (Soyyılmaz *et al*., 2021).

Therefore, the nutritional purpose of adding 3-FL to infant formula products is to create products that better reflect the compositional profile of human milk, to provide the same beneficial health effects to the developing infant. This is consistent with principles set forth by the Australia and New Zealand Food Regulation Ministerial Council's Policy Guideline on Infant Formula Products[6] regarding the composition of formula products for infants 0 to 12 months of age, as well as relevant Codex Standards for optional ingredients added to infant formulas and follow-up formulas for older infants (Codex Alimentarius, 2023a,b).

[6] https://www.foodregulation.gov.au/resources/publications/policy-guideline-infant-formula-products

A.2 Supporting Evidence

A.2.1 Biological Role of HMOs

Although HMOs share some common biological effects (*e.g.*, bifidogenic effect), they are structurally diverse molecules varying in number and type of monosaccharides and combinations of glycosidic linkages, resulting in HMOs having unique functions by structural class and individually (reviewed by Wichmann, 2024). As such, increased diversity of HMOs in infant formula, more closely reflecting the composition of this significant fraction in human milk, is expected to achieve greater health benefits in formula-fed infants, closer to that observed in breastfed infants (Wichmann, 2024).

Health effects of 3-FL have recently been reviewed in several publications (Li *et al.*, 2022; Du *et al.*, 2024; Wichmann, 2024). Findings from these reviews are summarised below, along with any new and relevant studies that have since been published.

3-FL supports the proliferation of beneficial gut bacteria

Similar to other HMOs, 3-FL has been demonstrated to promote the growth of beneficial bacteria *in vitro*, including numerous *Bifidobacterium spp.* and *Bacteroides spp.* (Yu *et al.*, 2013a,b; Garrido *et al.*, 2015; Thongaram *et al.*, 2017; Cheng *et al.*, 2020; Zabel *et al.*, 2020; Kong *et al.*, 2021; Salli *et al.*, 2021, 2023). A differentiating finding is that 3-FL was fermented by the microbiota at a slower rate compared to 2'-FL and DFL (Yu *et al.*, 2013a) or LNT2 (Kong *et al.*, 2021), and promoted a more diverse microbiota composition compared to LNT2 (Kong *et al.*, 2021). Fermentation of 3-FL by infant gut bacteria resulted in the production of lactate and short chain fatty acids, contributing to a favourable acidic environment that suppresses the growth of potentially pathogenic bacteria (Yu *et al.*, 2013a,b). The liberation of fucose from fucosylated HMOs such as 3-FL also contributes to the growth and cross-feeding of other commensal bacteria (Bunesova *et al.*, 2016; Engels *et al.*, 2016; Salli *et al.*, 2021).

3-FL has also been demonstrated to influence the composition of the gut microbiota and short-chain fatty acid profile in a mouse model (Holst *et al.*, 2022). In mice lacking infant-type bifidobacteria, 3-FL supplementation significantly increased the abundance of faecal *Bacteroidaceae* species compared to controls. The overall microbial composition was changed following 3-FL supplementation, also impacting short-chain fatty acid levels. When 3-FL supplementation was stopped, the abundance of *Bacteroidaceae* diminished already after one day, and continued to decrease throughout the remainder of the 7-day wash-out period.

The Bifidogenic effect of 3-FL has been substantiated in infant clinical trials (Holst *et al.*, 2023; Picaud *et al.*, 2023, 2024 [abstracts]). See Section A.2.2.

3-FL supports defence against infection

HMOs support the defence against pathogens *via* modulation of the immune system and by acting as decoy receptors. Interactions of HMOs with host cells and pathogens vary according to HMO class and individual HMOs, indicating structure-specificity (reviewed by Wichmann, 2024).

3-FL has been demonstrated *in vitro* to bind two different norovirus strains (Weichert *et al.*, 2016; Derya *et al.*, 2020), and to inhibit cell binding of the SARS-CoV-2 spike protein receptor binding domain that is crucial for the virus to enter host cells (Yu *et al.*, 2023).

3-FL but not 2'-FL alone had antiviral effects in H1N1 influenza virus mouse models (Moon *et al.*, 2024). 3-FL supplementation (alone or in combination with 2'-FL) increased the survival rate of H1N1-infected mice compared to vehicle controls and resulted in faster body weight recovery and lower viral titres. In lung sections of infected mice, infiltration of CD45-positive immune cells to infected regions was significantly higher in mice receiving 3-FL compared to controls. The mechanism of action was investigated in cell culture models. Incubation with 3-FL for several passages significantly reduced replication of the influenza virus in inoculated cells, and 3-FL had anti-viral effects against other viral strains and species tested in different cell types, including SARS-CoV-2. As cellular expression of interferon receptors increased in the absence of viral challenge, but the expression of interferon-stimulated genes was up-regulated only in the presence of viral challenge, 3-FL is thought to play a role in the modulation of the innate immune response against viral infection. In agreement, nitric oxide levels were significantly increased in cells treated with 3-FL following viral challenge, indicating activation of innate cellular immunity.

3-FL has also been demonstrated to inhibit the adhesion of bacterial pathogens in human cell lines. For example, pre-incubation of intestinal epithelial Caco-2 cells with 3-FL inhibited the adhesion of enteropathogenic *E. coli* O119 (Coppa *et al.*, 2006; Weichert *et al.* 2013). 3-FL also inhibited the adhesion of the respiratory pathogen *Pseudomonas aeruginosa* to Caco-2 cells as well as respiratory epithelial A549 cells (Weichert *et al.* 2013). This may be explained by 3-FL acting as a decoy receptor due to its strong affinity to the LecB lectin involved in *P. aeruginosa* biofilm formation and adherence of to host cells (Dupin *et al.*, 2018).

Infant clinical data support that 3-FL supplementation decreases the prevalence of opportunistic pathogens (Holst *et al.*, 2023; Picaud *et al.*, 2023, 2024 [abstracts]). See Section A.2.2.

3-FL supports intestinal barrier function

The ability of 3-FL and other HMOs to enhance intestinal barrier integrity has been studied *in vitro* (Boll *et al.*, 2024). Exposure of Caco-2 cells to 3-FL significantly enhanced transepithelial electrical resistance in a dose-dependent manner. In the presence of pro-inflammatory cytokines, 3-FL stabilised the transepithelial electrical resistance. 3-FL was among the HMOs tested with the most notable effects.

In an acute colitis mouse model, 3-FL improved symptoms and reversed the pathophysiology of the disease (Kim *et al*., 2023). Colitis was induced in mice by dextran sodium sulphate (DSS), a sulfonated polysaccharide that causes epithelial cell damage and disrupts intestinal barrier function (Eichele and Kharbanda, 2017). DSS-induced decreases in body weight and colon length were restored in mice administered 3-FL, and the Disease Activity Index (scored based on weight loss, stool condition, and gross bleeding) was improved. 3-FL reversed epithelial cell damage induced by DSS in colonic cell tissue of mice, by increasing villus height and thickness and decreasing the number of infiltrating inflammatory cells on the mucous membranes. Intestinal permeability was also improved in mice receiving 3-FL, as demonstrated by a reduction in fluorescently labelled dextran in the serum, and the reversal of changes in tight junction protein expression in the colon tissue of mice. Furthermore, 3-FL lowered serum levels of interleukin-6 and tumour necrosis factor-α induced by DSS. Similar results were obtained in an *in vitro* interleukin-6-induced cellular barrier dysfunction model. 3-FL restored epithelial paracellular permeability in Caco-2 cells in a dose-dependent manner, as well as tight junction protein expressions.

Clinical data support that 3-FL supplementation may help establish gut maturity during early infancy (Nori *et al*., 2024 [abstract]). See Section A.2.2.

A.2.2 Randomised Controlled Infant Clinical Study

5 HMOs (including 3-FL)

The beneficial effect on the gut microbiome of an infant formula supplemented with 5 manufactured HMOs (including 3-FL) during the first 4 months of life has been evaluated in a double-blind, randomised, controlled, multicentre[7] clinical trial (Holst *et al*., 2023; Clinical Trial Registry NCT03513744). Growth, safety and tolerability outcomes of this clinical trial reported by Parschat *et al*. (2021) were previously evaluated by FSANZ during their assessment of Application A1265 (FSANZ, 2023b). As Holst *et al*. (2023) was published after the completion of FSANZ's assessment of Application A1265 (FSANZ, 2023a), the results summarised below.

Briefly, the study consisted of a 4-month intervention period, followed by a 2-month voluntary follow-up period. Healthy term infants ≤14 days of age exclusively consuming infant formula were randomised to receive the Control Formula (CF) without HMOs or the Test Formula (TF) with 5.75 g/L of total HMOs (2'-FL, 3-FL, LNT, 3'-SL, and 6'-SL). In parallel, a group of exclusively breastfed (BF) infants were enrolled as a reference group.

The faecal microbial composition was evaluated in 311 infants who participated in the study. Faecal samples were collected at Weeks 1, 2, 4, 8, 12, and 16 and 6 months after enrolment—no samples were collected at baseline before the start of intervention.

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

The microbial composition was significantly different in TG compared to CG at all time points (except Week 8), and more closely resembled that of BF at Weeks 1, 12, and 16. The relative abundance of certain genera were significantly different in TG compared to CG: increased *Bifidobacterium* (Weeks 1 to 4 and Week 16); increased *Bacteroides* (Weeks 1, 8 and 12); and decreased *Enterococcus* (Weeks 1 and 4). *Bifidobacterium* species expressing aromatic lactate dehydrogenase able to produce aromatic lactic acids (*e.g.*, *via* the degradation of aromatic amino acids such as tyrosine) were dominant in BF and were also significantly more abundant in TG compared to CG at Weeks 1 and 16. Furthermore, the relative abundance of prevalent opportunistic pathogens (generally) was significantly lower in TG compared to CG at Weeks 1 to 4, as was *Clostridioides difficile* (specifically) at Week 16, though none of the species identified were associated with toxigenicity.

Next, the metabolic capability of the microbiome was assessed by Gut Metabolic Modules. Metabolic modules for mucin, starch, and tyrosine degradation were enriched in both BF and the TG compared to CG. Specifically, bacterial mucin degradation was significantly enriched at Weeks 1 to 4 and tyrosine degradation was significantly enriched at Weeks 1 and 16 in TG compared to CG. Contrary, lipid, amino acid, and monosaccharide degradation were enriched in the CG compared to TG and BF.

The study authors concluded that infant formula supplemented with the 5 HMOs shifts the composition of the infant gut microbiome closer to that of breastfed infants compared with infants receiving infant formula without HMOs.

6 HMOs (including 3-FL) plus 2 Probiotics

The gut microbiome modulating effects of an infant formula supplemented with 6 manufactured HMOs (including 3-FL) and 2 probiotics has been evaluated in a randomised, controlled, double-blind trial conducted across 18 sites in Belgium, Spain, France, and Germany in infants through to 15 months of age (Miranda *et al.*, 2024 [abstract]; Noti *et al.*, 2024 [abstract]; Picaud *et al.*, 2023, 2024 [abstracts]; Clinical Trial Registry NCT04962594). The full clinical study report is provided in Appendix 8.

Healthy term infants ≤ 14 days of age were enrolled in the study. Infants exclusively consuming and tolerating cow's milk infant formula were randomised to the Control Formula (CF) or Experimental Formula (EF). A non-randomised parallel reference group of exclusively breastfed (BF) infants was also enrolled.

The age-specific formulas contained nutrients in amounts intended for full nutritional support of infants 0 up to 6 months of age (starter infant formula), infants 6 up to 12 months of age (follow-up formula), and young children 12 up to 15 months of age (growing up milk). Study formulas consisted of a partially hydrolysed 100% whey-based formula. The CF did not contain added HMOs or probiotics. EFs were supplemented with the 6 manufactured HMOs (2'-FL, DFL,

3-FL, LNT, 3'-SL, and 6'-SL) that replaced part of the lactose content and *B. infantis* LMG11588 plus *B. Lactis* CNCM I-3446 probiotics. Target amounts of each HMO are presented in Table 4.

Table 4 Target Levels[a] of HMOs in Age-Specific Experimental Formulas

HMO	Starter Infant Formula (0 to 6 months)	Follow-Up Formula (6 to 12 months)	Growing Up Milk (12 to 15 months)
2'-Fucosyllactose (g/L)	0.87	0.26	0.21
Difucosyllactose (g/L)	0.12	0.04	0.03
3-Fucosyllactose (g/L)	0.24	0.26	0.29
Lacto-*N*-tetraose (g/L)	0.29	0.15	0.08
6'-Sialyllactose (g/L)	0.15	0.05	0.04
3'-Sialyllactose (g/L)	0.11	0.11	0.11
Total HMO (g/L)	**1.77**	**0.87**	**0.75**

HMO = human milk oligosaccharide.

[a] Target levels reflect what is expected analytically in the formulas, considering added HMOs, innate HMOs from other ingredients, and process loss. Values have been rounded to the nearest hundredth (two decimal places).

In addition to the demonstration of age-appropriate growth (primary objective – see Section C.2.2), the key secondary objective of the trial was to demonstrate the Bifidogenic effect of a combination of 6 HMOs and 2 probiotics in a starter infant formula by showing increased *Bifidobacteria* abundance with EF compared to CF at 3 months of age. Other benefit-related secondary outcomes evaluated at different stages of feeding include faecal microbiome, faecal metabolic profile, faecal markers of immune and gut health, and blood markers of immune health. Primary and secondary objectives of the trial were analysed in the full analysis set (FAS)[8] and per protocol set (PP)[9], with a special subset of PP (sub-PP)[10] analysed for gut microbiome outcomes. Other secondary outcomes were analysed in FAS.

A total of 318 infants were enrolled in the trial (119 in EF, 117 in CF, and 82 in BF). The FAS included 313 infants (118 in EF, 114 in CF, and 81 in BF). Excluded from FAS (n=5) were randomised subjects who never took any of the assigned product (study formula or breastmilk), subjects who received incorrect study formula, or subjects who failed to satisfy study entry eligibility criteria. Infants in FAS were analysed according to the assigned product group per randomisation. The PP included 227 infants (84 in EF, 84 in CF, and 59 in BF), after excluding subjects in FAS with major protocol violations influencing the primary endpoint (weight gain velocity).

[8] All subjects who met the study inclusion and exclusion criteria and who received and consumed their allocated product (for CF and EF subjects) or who consumed breastmilk (for BF subjects).
[9] All subjects in the FAS population who adhered to all protocol requirements without any major protocol deviations influencing the primary endpoint (weight gain velocity) and with complete baseline data and at least one post-randomization data point.
[10] All subjects in the PP population without minor protocol deviations which may have impacted the microbiome related outcomes.

Bifidobacterium abundance was significantly higher in EF compared to CF at 3 and 6 months in FAS, PP, and sub-PP populations. Specifically at 3 months, the relative abundance of infant-type *Bifidobacterium* (*B. longum subsp. longum*, *B. infantis*, *B. breve*, *B. bifidum*, *and B. scardovii*) was significantly higher in EF compared to CF. The prevalence of *B. lactis* and *B. infantis* significantly expanded from baseline to 6 months in EF, with *B. infantis* LMG11588 highly prevalent in EF. In contrast, the prevalence of toxigenic *Clostridiodes difficile*, a potentially pathogenic bacterium, was significantly lower in EF compared to CF at 3 and 6 months.

Faecal pH was significantly lower in EF compared to CF at 3 months, though SCFAs were similar between EF and CF at all timepoints. Faecal alpha-1 antitrypsin (a marker of intestinal permeability) was also significantly lower in EF compared to CF at 3 months, indicating that the test infant formula positively supported gut maturity during early infancy. There was no significant difference in faecal secretory immunoglobulin A (sIgA) and calprotectin levels between EF and CF. At 4 months, blood T-helper 2 cell levels were significantly lower in EF compared to CF and comparable to BF, and T-helper 1 cells in EF but not CF were positively correlated with specific T-helper cell subsets, suggesting a balanced cross-regulation potentially contributing to immune homeostasis.

The study authors concluded that the formulas supplemented with the 6 HMOs (including 3-FL) and 2 probiotics positively supports the gut environment by increasing beneficial bacteria (particularly *Bifidobacteria*) and decreasing pathogenic bacteria, and positively supports gut maturity during early infancy.

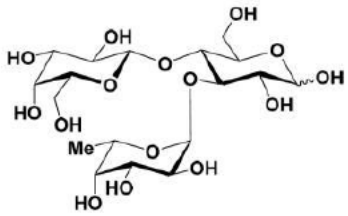
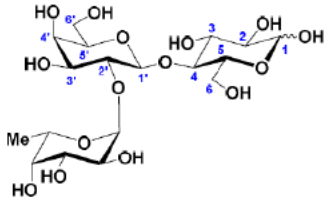
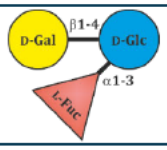
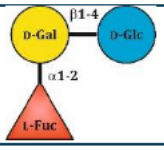
B. Technical information on the use of the nutritive substance

B.1 Identification

3-FL obtained from microbial fermentation is chemically and structurally identical to 3-FL that is naturally present in human breast milk, as confirmed by 1D-^{1}H, 1D-^{13}C and 2D nuclear magnetic resonance (NMR) spectroscopy. A comparison 3-FL obtained from microbial fermentation to 3-FL isolated from breast milk is provided in Appendix 3 **(Confidential Commercial Information)**.

3-Fucosyllactose (3-FL) is a trisaccharide consisting of D-glucose, D-galactose, and L-fucose. It is derived from lactose by addition of a fucose sugar to the glucose unit of lactose by an *alpha* (1→3) linkage. 3-FL is a simple structural isomer of 2′-FL; for 2′-FL, the fucose sugar is linked to the galactose unit of lactose by an *alpha* (1→2) linkage. As per HMO nomenclature, there is no prime symbol in the ingredient name when the fucose unit is linked to the glucose unit of lactose (*i.e.*, 3-FL); the prime symbol is included only when the fucose unit is linked to the galactose unit of lactose (*i.e.*, 2'-FL) (McNaught, 1996). A comparison of the 3-FL and 2′-FL structures is provided in Table 5.

Table 5 Comparison of 3-FL and 2′-FL Structures

	3-FL	2′-FL
Molecular Structure		
Symbol Nomenclature		
		

2'-FL = 2'-fucosyllactose; 3-FL = 3-fucosyllactose.

Further details on the identity of 3-FL are provided in Table 6.

Table 6 Identity of 3-Fucosyllactose

Generic Product Name	3-Fucosyllactose
Common Abbreviations	3-FL; 3FL
Synonyms	3-*O*-Fucosyllactose, 3-*O*-L-Fucosyl-D-lactose, 3-Fucosidolactose; "Lewis^{X}-2g" (= glucose analogue of histo-blood group Lewis^{X} antigen)
IUPAC Name	β-D-Galactopyranosyl-(1→4)-[α-L-fucopyranosyl-(1→3)]-D-glucose
IUPAC Abbreviation (extended)	β-D-Gal*p*-(1-4)-[α-L-Fuc*p*-(1-3)]-D-Glc
IUPAC Abbreviation (condensed)	Gal-(β1-4)-[Fuc-(α1-3)]-Glc
Molecular Formula	$C_{18}H_{32}O_{15}$
Molecular Mass	488.44 g/mol
CAS Number	41312-47-4
CAS Name	*O*-6-Deoxy-α-L-galactopyranosyl-(1→3)-*O*-[β-D-galactopyranosyl-(1→4)]-D-glucose

CAS = Chemical Abstracts Service; IUPAC = International Union of Pure and Applied Chemistry.

B.2 Chemical and Physical Properties

3-FL produced by microbial fermentation is a white to off-white amorphous powder or agglomerates. It is readily soluble in aqueous solutions (maximum 400 mg/mL, 25 °C), with poor solubility in any organic solvents.

The bulk stability of the 3-FL ingredient has been evaluated in a 2-year accelerated stability study (40°C, 75% relative humidity), and is being evaluated in an ongoing 5-year real-time stability study (25°C, 60% relative humidity). There have been no appreciable changes in the organoleptic properties of the ingredient, no appreciable degradation of 3-FL, no changes in the impurity profile, and no alterations in the microbiological quality of the ingredient under these storage conditions.

The stability of the 3-FL ingredient has also been investigated in a commercially representative infant formula food matrix formulated with 3-FL at a final concentration of 1 g/100 g powder using wet blending techniques and processed using standard industry steps including pasteurisation, homogenisation, spray-drying, and canning in gas-flushed cans. The powdered infant formula cans were stored at temperatures of 5, 25, 30, and 37°C, and sampled at regular time points over a period of 3 years, to evaluate select compositional and microbiological quality indicators.

Overall, the results indicate that the manufactured 3-FL is expected to be stable over its intended shelf-life of 5 years and is stable under its intended conditions of use in infant formula products. These data are consistent with those of other HMOs manufactured by Glycom.

Analytical results of the real-time and accelerated bulk stability studies, as well as the stability study in the representative powdered infant formula food matrix, are provided in Appendix 4 **(Confidential Commercial Information)**.

The 3-FL ingredient was also subjected to stress stability studies to identify potential degradation products, including:

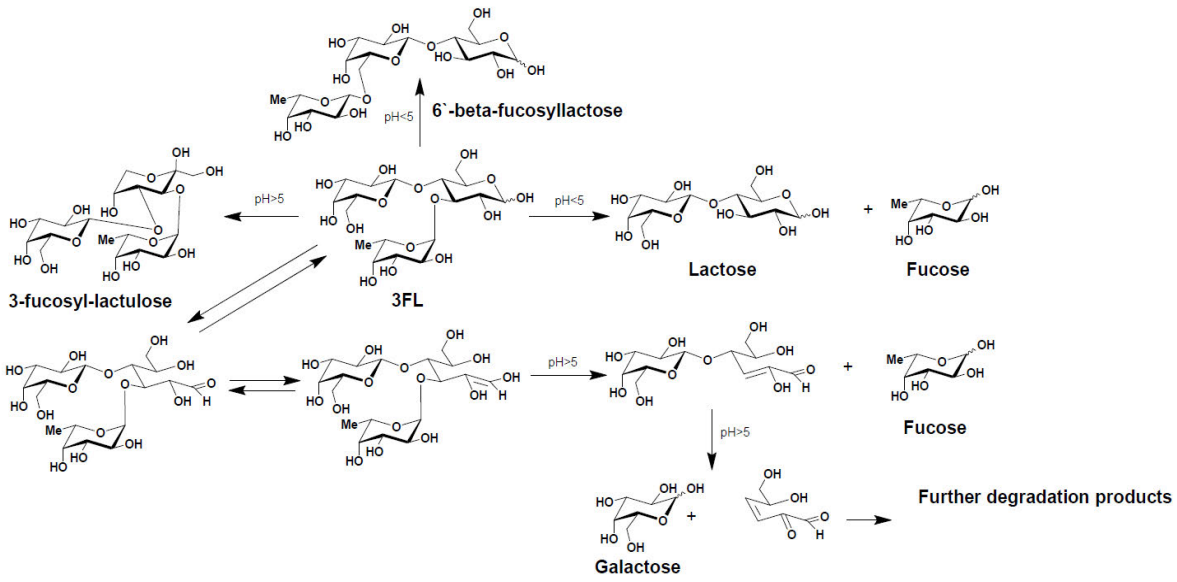
- 28-day solid state forced thermal stability study (80°C, under dry and humid conditions);
- Up to 28-day stability in aqueous solution at six different pH levels (3.0, 5.0, 6.8, and 9.0; in 0.1 N HCl; and in 0.01 N NaOH) and without added buffer; and
- 24-hour stability in the presence of two different oxidizing agents: hydrogen peroxide and 4,4’-azobis-(4-cyanovaleric acid).

Four potential pH-dependent degradation pathways were identified. The first two pathways are comprised of 3-FL hydrolysis to lactose and fucose and its isomerisation to 6'-β-fucosyllactose—these are the main degradation pathways when the ingredient is in solution at

pH below 5. The third pathway involves isomerisation to 3-fucosyl-lactulose and occurs when the pH is above 5.

The fourth pathway is more complex; it corresponds to the "peeling reaction," characteristic to carbohydrates substituted in position 3 related to the terminal anomeric carbon. The primary products detected are fucose and galactose in roughly equal amounts, with the glucose moiety going on to form further degradation products. The proposed degradation pathways are depicted in Figure 1. The stability data indicate that 3-FL is most stable in aqueous solution at pH 5.

Figure 1 Degradation Pathways of 3-FL in Aqueous Solutions at Varying pH



No other potential degradation pathways were identified. Results are available upon request.

B.3 Impurity Profile

Glycom's 3-FL is a highly purified ingredient specified to contain at least 90 w/w% of 3-FL on a dry weight basis. The ingredient also contains small quantities of well-characterised carbohydrates, including D-lactose (starting substrate) and other related carbohydrates produced during the fermentation process. Food grade specifications that have been established for Glycom's 3-FL, informing on the composition of the ingredient, are described in Section B.5.1.

Additional quality control measures include the confirmation of absence of a range of potential residual compounds and trace elements. These include amino acids and biogenic amines, the production organism and its DNA, anions and trace elements, and heavy metals. As these have been confirmed to be absent in the 3-FL ingredient, they are not proposed for addition to the product specifications.

B.3.1 Absence of Amino Acids and Biogenic Amines

3-FL is secreted into the fermentation broth, and the production organism is efficiently removed at the end of the fermentation process. Nevertheless, as a precautionary measure, production batches have been analysed for secondary metabolites and cellular components that may potentially originate from the fermentation medium. Results of analyses of the 3-FL ingredient for biogenic amines (histamine, tyramine, spermidine, cadaverine, and putrescine) and amino acids and their metabolites (glutamic acid, arginine, histidine and *gamma*-aminobutyric acid) did not identify significant detectable levels of these contaminants in any of the manufacturing batches of the finished ingredient. Certificates of Analysis demonstrating the absence of these parameters can be provided upon request.

B.3.2 Absence of Production Organism and its DNA

The production microorganism is efficiently removed by the ultrafiltration at the end of the fermentation process during the upstream processing. Additionally, various sequential filtration and purification processes are applied during the downstream processing to ensure the final purity of the 3-FL ingredient.

Product specifications for 3-FL include acceptable limits for residual protein (≤ 0.01 w/w %) and residual endotoxins (≤ 10 EU/mg) (see Section B.5.1). Results from batch analyses demonstrate that any residual levels are well below the specification limit for all batches of 3-FL (see Section B.5.2). Furthermore, absence of the *E. coli* K-12-derived production microorganism in the 3-FL ingredient is demonstrated by testing 3-FL batches for bacteria from the *Enterobacteriaceae* family according to an internationally recognised method (ISO 21528-1).

Finally, the absence of the production organism in the finished ingredient is also supported by analyses for residual DNA in batches of 3-FL. The absence of residual DNA from the production organism is confirmed by three different validated quantitative polymerase chain reaction (qPCR) methods. These qPCR methods target short sub-sequences of inserted genes from donor organisms (including the gene encoding the enzyme for 3-FL biosynthesis), as well as a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. Analyses of four batches of 3-FL demonstrate no detectable levels of residual DNA (limit of quantification of 4 µg/kg) in the final ingredient (data available upon request).

B.3.3 Minerals and Trace Elements

Due to the nature of the fermentation process, 3-FL may potentially contain trace elements and minerals (as carry-over from the fermentation medium). However, the use of nanofiltration and ion-exchange purification is sufficient to reduce any appreciable carry-over of minerals and trace elements from fermentation into the final ingredient. The results of trace element analyses of four batches of 3-FL confirm that these are not present to any relevant degree (data available upon request).

It should be emphasised that finished infant formula products containing 3-FL will comply with compositional requirements for infant formula and follow-on formula established in Standard 2.9.1 of the Code.

B.3.4 Heavy Metals

Analyses for heavy metals were conducted on four batches of 3-FL. Results demonstrate that lead, arsenic, cadmium, and mercury are below specification limits prescribed in Schedule 3 under Section S3—4 of the Code. Results from batch analyses for heavy metal are provided in Appendix 5 **(Confidential Commercial Information)**.

B.4 Manufacturing Process

Glycom's 3-FL is manufactured in compliance with current Good Manufacturing Practice (cGMP) and the principles of Hazard Analysis Critical Control Point (HACCP). The manufacturing process of 3-FL is largely comparable to the manufacturing process of Glycom's other HiMOs already permitted for use as nutritive substances in infant formula products in Australia and New Zealand[11]. A schematic overview of the manufacturing process for 3-FL is presented in Table 7 below.

The manufacturing process can be broadly divided into 2 stages:

In Stage 1 [Upstream Processing (USP)], D-lactose is converted to 3-FL by the adapted cellular metabolism of the production microorganism, which uses D-glucose (or alternatively glycerol or D-sucrose) as an energy and carbon source. Fermentation is performed in a chemically defined, salt-based, minimal growth medium. The production microorganism is removed from the fermentation medium at the end of the fermentation process.

In Stage 2 [Downstream Processing (DSP)], a series of purification, isolation, and concentration steps are used to generate the final high-purity 3-FL ingredient.

Each of these stages are described further in Appendix 6 (**Confidential Commercial Information**).

[11] 2'-Fucosyllactose, lacto-N-neotetraose, a combination of 2'-fucosyllactose and difucosyllactose, lacto-N-tetraose, 6'-sialyllactose sodium salt and 3'-sialyllactose sodium salt sourced from *Escherichia coli* K-12 (Schedule 26).

Table 7 Overview of the Manufacturing Process of 3-FL

Stage 1		Upstream Processing (USP)
Steps	1	Media Preparation
	2	Propagation
	3	Seed Fermentation
	4	Main Fermentation Phases:
	4A	Growth (Batch) Phase (optional)
	4B	Production (Fed-Batch) Phase
	4C	Harvest/Storage of Culture Broth
	5	Removal of Microorganism
Stage 2		**Downstream Processing (DSP)**
Steps	6	Purification/Concentration 1
	7	Ion Removal
	8	Decolourisation
	9	Purification/Concentration 2
	10	Drying
	11	Sampling and Packaging
	12	Quality Control and Batch Release

3-FL = 3-fucosyllactose.

All processing aids, raw materials, unit operations, and filter aids have been sourced considering a range of strict food quality requirements. Raw materials and processing aids used in the production of 3-FL are provided in Appendix 6 **(Confidential Commercial Information)**.

Both manufacturing stages (USP and DSP) are controlled by a HACCP plan which includes specifications for equipment, raw materials, product, and packaging materials. Master operating instructions are followed, batch records are kept, a number of in-process controls are applied, and the 3-FL product is controlled by Certificates of Analysis and batch release routines. The manufacturing process (including all used processing aids, raw materials, unit operations and filter aids) as well as the food safety management system comply with the Food Safety Systems Certification 22000 (FSSC 22000) and the 2015 International Organisation for Standardisation 9001 (ISO 9001:2015).

3-FL is manufactured in Denmark under contained use of the genetically modified microorganism (following strictly the Danish implementation of Directive 2009/41/EC of the European Parliament and of the Council of 06 May 2009 on the contained use of genetically modified microorganisms). Thus, the production organism used in the manufacturing process of 3-FL will not enter Australia or New Zealand.

B.5 Specification for Identity and Purity

B.5.1 Specifications

Food grade specifications, presented in Table 8, have been established for Glycom's 3-FL. These specifications have been accepted in other jurisdictions where Glycom's 3-FL is already permitted for use, including the EU (EU, 2023), the UK (UK Government, 2024a,b,c) and the U.S. (GRN 1037 – U.S. FDA, 2022). All methods of analysis are either internationally recognised or developed and validated internally by Glycom and confirmed by independent accredited external laboratories. Details of the internal methods of analysis are provided in Appendix 7 **(Confidential Commercial Information)**.

Table 8 Specifications for 3-FL

Parameter	Specification	Method
Appearance	Powder, agglomerates, powder with agglomerates	ISO 6658
Colour	White, white to off-white, off-white	ISO 6658
Identification by RT	RT of main component corresponds to RT of standard ± 3%	Glycom method HPLC-402-4C4-001
Assay (water-free) specified saccharides[a]	≥ 92.0 w/w %	Glycom method HPLC-402-4C4-001, HPAEC-HMO-021, and HPLC-3FL-003
Assay (water-free) 3-Fucosyllactose	≥ 90.0 w/w %	Glycom method HPLC-402-4C4-001
L-Fucose	≤ 1.0 w/w %	Glycom method HPLC-3FL-003
D-Lactose	≤ 5.0 w/w %	Glycom method HPAEC-HMO-021
3-Fucosyllactulose	≤ 1.5 w/w %	Glycom method HPAEC-HMO-021
Sum of other carbohydrates	≤ 5.0 w/w %	Glycom method HPAEC-HMO-021 and HPLC-3FL-003
pH in 5% solution (20°C)	3.2 to 7.0	Ph. Eur. 9.2 2.2.3
Water	≤ 6.0 w/w %	Glycom method KF-001
Ash, sulphated	≤ 0.5 w/w %	Ph. Eur. 9.2 2.4.14
Residual protein by Bradford assay	≤ 0.01 w/w %	Glycom method UV-001
Residual endotoxins	≤ 10 E.U./mg	Ph. Eur. 2.6.14
Lead	≤ 0.05 mg/kg	EN 13805; EPA-6020A
Microbiological Specifications		
Aerobic mesophilic total plate count	≤ 1,000 CFU/g	ISO 4833-1 or ISO 4833-2
Enterobacteriaceae	Absent in 10 g	ISO 21528-1

Parameter	Specification	Method
Salmonella	Absent in 25 g	ISO 6579 or AFNOR BRD 07/11-12/05
Yeasts	≤ 100 CFU/g	ISO 21527-2
Moulds	≤ 100 CFU/g	ISO 21527-2

3-FL = 3-fucosyllactose; CFU = colony-forming units; EPA = Environmental Protection Agency; E.U. = endotoxin units; HPAEC = high-performance anion-exchange chromatography; HPLC = high-performance liquid chromatography; ISO = International Organization for Standardization; KF = Karl-Fischer; LC-MS/MS = liquid chromatography coupled with tandem mass spectrometry; Ph. Eur. = *European Pharmacopeia*; RT = retention time; UV = ultraviolet.
[a] Specified saccharides include 3-FL, D-lactose, L-fucose, and 3-fucosyl-lactulose.

B.5.2 Batch Analyses

Analyses have been conducted on 5 independent representative batches of 3-FL. Certificates of Analysis are provided in Appendix 5 **(Confidential Commercial Information)**. The results demonstrate that the 3-FL complies with the established product specifications defined in Section B.5.1.

B.6 Analytical Method for Detection

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

B.7 Proposed Food Label

Infant formula products containing 3-FL will be labelled in accordance with applicable provisions in the Code established under Part 1.2 of on labelling and other information requirements and in Standard 2.9.1 for infant formula products.

Specifically, as per Standard 1.2.4 of the Code, 3-FL is intended to be listed as an ingredient on the statement of ingredients of infant formula products containing 3-FL.

Infant formula products containing 3-FL are also intended to be labelled in accordance with the specific labelling provisions for nutritive substances for this type of special purpose foods, established in Standard 2.9.1 of the Code.

Furthermore, as per prohibited representations in Section 2.9.1—24(ca) or (cb) of the Code, the words 'human milk oligosaccharide' or 'human milk identical oligosaccharide', and the abbreviations 'HMO' or 'HiMO', and any other word(s) or abbreviation having the same or similar effect, will not be used to identify 3-FL on the label or package.

As the production microorganism is removed during the manufacturing process, as evidenced by non-detectable levels of residual DNA in the final ingredient (< 4 µg/kg), and any novel

proteins are efficiently removed and controlled by a specification limit for residual proteins (≤ 0.01 w/w %), it is considered that labelling requirements according to Section 1.5.2—4 for genetically modified food do not apply to 3-FL, similar to other HMOs manufactured by microbial fermentation.

C. Information Related to the Safety of the Nutritive Substance

The general basis of the approach of this safety assessment is the demonstration that:

(1) The 3-FL manufactured using gene technology is well characterised and confirmed to be chemically and structurally identical to 3-FL found in human milk. 3-FL is intended to be added alone or in combination with other manufactured HMOs to infant formula products at levels similar to those in human milk.

(2) 3-FL is a highly purified ingredient (≥ 90 w/w% on water-free basis), and the remaining composition of the ingredient is well characterised and controlled by well-established specifications.

(3) The production organism is well characterised and is confirmed to be effectively removed during the manufacturing process.

(4) Corroborative safety studies, including those conducted in animals and humans, demonstrate that the manufactured 3-FL does not pose any safety concerns.

(5) Other HMOs manufactured by Glycom using the same platform strain have been approved for use in infant formula products in Australia and New Zealand (2'-FL, LNnT, 2'-FL/DFL, LNT, 3'-SL sodium salt and 6'-SL sodium salt).

(6) Glycom's 3-FL has been approved for use and commercialised in infant formula products in other jurisdictions (EU, UK and USA).

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

The manufactured 3-FL is chemically and structurally identical to 3-FL in human milk (see Appendix 3 - **Confidential Commercial Information**). As such, the absorption, distribution, metabolism, and excretion (ADME) of the manufactured 3-FL is expected to be the same as 3-FL from human milk.

The ADME of HMOs have been investigated in a number of studies (Brand-Miller *et al.*, 1995, 1998; Rudloff *et al.*, 1996, 2006, 2012; Obermeier *et al.*, 1999; Engfer *et al.*, 2000; Gnoth *et al.*, 2000; Chaturvedi *et al.*, 2001; Coppa *et al.*, 2001; Rudloff and Kunz, 2012; De Leoz *et al.*, 2013;

Dotz *et al.*, 2014, 2015; Goehring *et al.*, 2014; Kunz and Rudloff, 2017). These studies suggest that HMOs do not undergo any significant digestion in the upper gastrointestinal tract but are instead fermented in the colon by intestinal microbiota or are excreted unchanged in the faeces. A small proportion of ingested HMOs may be absorbed intact, with approximately 1 to 2 % of the total amount of HMO ingested being excreted unchanged in the urine.

Consistent with these publications, in EFSA's Scientific Opinion on the essential composition of infant and follow-on formula, the EFSA NDA Panel considers HMOs as "non-digestible oligosaccharides" (EFSA, 2014). Furthermore, in their Scientific Opinion on the safety of Glycom's 3-FL, the EFSA NDA Panel affirmed that there is limited digestion and absorption of the novel food in the upper gastrointestinal tract, and that HMOs are fermented by intestinal microbiota in the colon, with a fraction excreted unchanged in the faeces and a small fraction in the urine (EFSA, 2023).

C.2 Safety Studies

C.2.1 Toxicity Studies

Glycom's 3-FL had been evaluated in a comprehensive series of toxicological studies, including a bacterial reverse mutation assay, an *in vitro* mammalian cell micronucleus test in human lymphocytes, and an adapted sub-chronic (90-day) oral toxicity study in neonatal rats. The 3-FL test article (composed of 94.6 w/w% of 3-FL on a water-free basis) was representative of the material intended to be commercially marketed. The studies were performed in accordance with the Organisation for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) (OECD, 1998) and appropriate OECD test guidelines where applicable.

Consistent with the expected safety of HiMOs that is established from their history of consumption in human milk, the results of the toxicity studies demonstrate that Glycom's 3-FL does not pose any toxicological concerns.

Detailed descriptions of these studies are presented below. The studies have been published (Phipps *et al.*, 2022). A copy of the full unpublished study reports is provided in Appendix 8 **(Confidential Commercial Information)**.

Repeated-Dose Studies

14-Day Toxicity Study in the Neonatal Rat

A 14-day repeat dose toxicity study was conducted in rats to evaluate the potential short-term toxicity of 3-FL and select dose levels for the subsequent 90-day study (Stannard, 2021a [unpublished]; Phipps *et al.*, 2022).

Groups of eight male and eight female neonatal rats were dosed with 0 (water for injection), 3,000, or 4,000 mg/kg body weight/day, by oral gavage at a dose volume of 10 mL/kg body weight, once daily for 14 days, until the day before necropsy. The 3-FL formulations were prepared correcting for 3-FL content; thus, in terms of test item as supplied (accounting for "other carbohydrates" within the test item batch), doses were equivalent to 0, 3,240, or 4,320 mg/kg body weight/day. The high dose was the maximum feasible dose based on solubility in the vehicle.

All animals were weighed and observed daily for changes in clinical condition, with detailed observations performed before, shortly after, and at 1 to 2 hours after dosing; a final observation was also made towards the end of the working day. At the end of the dosing period, animals were subjected to a gross necropsy.

There were no premature deaths and no adverse clinical signs. The only noteworthy clinical signs were skin reddening around the anus/perianal region in males and females in both groups receiving 3-FL between Days 8 and 12 of age (Days 2 to 6 of dosing), with associated yellow staining around the anus/perianal region between Days 10 and 21 of age (Days 4 to 15 of dosing) and a low incidence of encrustation around the perianal region in treated females between Days 11 and 13 of age (Days 5 to 7 of dosing). In addition, 1 female given 3,000 mg/kg/day showed red discharge from the perianal region on Days 11 and 12 of age (Days 5 and 6 of dosing, respectively). Since these clinical signs were transient and were largely absent by the end of the study, they were considered non-adverse.

There were no biologically relevant differences in body weight between test item groups and controls, nor were there any test item–related macroscopic abnormalities at necropsy. Administration of 3-FL at 3,000 or 4,000 mg/kg body weight/day, by gavage to neonatal rats once daily for 14 days from Day 7 of age, was well tolerated and did not result in any adverse test item–related effects. It was therefore concluded that the high dose of 4,000 mg/kg body weight/day (the maximum feasible dose) was the no-observed-adverse-effect level (NOAEL) and would be a suitable high dose for the 90-day study.

90-Day Toxicity Study in the Neonatal Rat

A 90-day repeat dose toxicity study was conducted to evaluate the potential subchronic toxicity of 3-FL when administered orally, by gavage, to neonatal rats from Day 7 of age (Stannard, 2021b [unpublished]; Phipps *et al.*, 2022). The study was designed based on OECD Test Guideline 408 (OECD, 2018), but adapted to start dosing in neonatal animals to consider toxicity testing requirements for new chemicals intended for use in the paediatric population (ICH, 2020).

Groups of 10 male and 10 female neonatal Crl:CD(SD) rats received 0 (vehicle: water for irrigation), 1,000, 2,000, or 4,000 mg/kg body weight/day 3-FL by oral gavage at a dose volume of 10 mL/kg body weight, once daily for 90 days, until the day before necropsy. An additional

reference control group (comprising the same number of animals) received oligofructose powder (a non-digestible oligosaccharide permitted in infant nutrition) at 4,000 mg/kg body weight/day, under the same conditions, to allow for direct comparison against the high-dose 3-FL group and identify any effects related to the general fibre-like characteristics of the reference material. Doses of 3-FL and the reference control were corrected to account for "other carbohydrates" within the test article batches (thus, the high dose corresponded to a total carbohydrate amount of 4,320 mg/kg body weight/day). A further five males and five females in the vehicle control, with high-dose 3-FL and reference control groups, were also dosed once daily for at least 90 days and then kept undosed for 4 weeks to assess the reversibility of any observed effects seen in the dosing period.

Animals were examined daily from the start of treatment. Body weights were recorded daily from the start of dosing until weaning and twice weekly thereafter. Food intake was recorded twice weekly from weaning until necropsy. The eyes of vehicle control, reference control, and high-dose animals were examined in Week 13. Blood samples were collected and analysed for haematology, blood chemistry, and thyroid hormone (triiodothyronine [T3], thyroxine [T4], and thyroid-stimulating hormone [TSH]) during Week 13. Urine samples were collected and analysed for urinalysis in Week 13 and at the end of the recovery period.

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

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

There were no deaths and no test item–related clinical signs, nor were there any ocular findings at the ophthalmic examinations in Week 13 of dosing. Body weight and food consumption were unaffected by 3-FL administration. There were no effects of 3-FL on the age or body weight at which the males and females attained physical signs of sexual maturation (balano-preputial skinfold separation or vaginal opening for males and females, respectively), nor on age of attainment of the surface and air righting reflexes, performance in the pupil reflex and startle response tests, or mean ulna growth. Behaviour of the animals during the in-hand and arena observations, as well as Morris maze performance, were similar across all groups. Oestrous cycles were unaffected by 3-FL administration, with most females in all groups showing an oestrus smear prior to termination.

No test item–related adverse effects on haematology, clinical biochemistry, or urinalysis parameters were observed. A statistically significant decrease in specific gravity was observed in all male 3-FL groups, in females given 2,000 or 4,000 mg/kg body weight/day, and in reference control males and females. In addition, males given 4000 mg/kg body weight/day showed a slight, but statistically significant increase in urinary pH, and a decrease in protein, creatinine, and glucose concentrations (both in total and concentration terms). However, reference control males also showed an increase in urine volume, and a decrease in urinary protein, glucose, and creatine concentration when compared to vehicle controls. No differences in these parameters were observed at the end of the 4-week recovery period. No test article-related differences in organ weights were observed and there were no 3-FL-related macroscopic or histopathological abnormalities; the only findings observed were incidental and generally consistent with changes encountered in Sprague-Dawley rats of this age kept under laboratory conditions.

The results to date demonstrate that once daily oral gavage administration of 3-FL to neonatal Crl:CD(SD) rats for 90 days (from Day 7 of age) at doses up to 4,000 mg/kg body weight/day (total carbohydrate amount of 4,320 mg/kg body weight/day) was well tolerated and not associated with any test article-related adverse effects.

Genotoxicity Studies

<u>Bacterial Reverse Mutation Test</u>

The potential mutagenicity of 3-FL was evaluated in a bacterial reverse mutation test (Gilby, 2021a [unpublished]; Phipps *et al.*, 2022). The study was conducted in compliance with OECD Test Guideline 471 (OECD, 1997).

Two separate tests (plate incorporation assay and pre-incubation assay) were conducted using *Salmonella* Typhimurium strains TA98, TA100, TA1535, and TA1537 and *E. coli* strain WP2 uvrA (pKM101), which were exposed to 3-FL at concentrations of up 5,000 µg/plate (the OECD Test Guideline 471 maximum recommended concentration) in the absence and presence of external metabolic activation (S9 mix).

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

There was no evidence of mutagenicity following exposure to 3-FL in either test, in the absence or presence of metabolic activation. In contrast, the positive controls induced biologically relevant increases in revertant colony counts (with metabolic activation where required), which demonstrated the sensitivity of the assay and metabolic activity of the S9 preparations. It was concluded, therefore, that 3-FL is non-mutagenic at concentrations up to 5,000 µg/plate (the OECD Test Guideline 471 maximum recommended concentration).

In Vitro Mammalian Cell Micronucleus Test

The clastogenic and aneugenic potential of 3-FL was evaluated in an *in vitro* mammalian cell micronucleus test, conducted using human lymphocytes (Gilby, 2021b [unpublished]; Phipps *et al.*, 2022). The study was conducted in compliance with OECD Test Guideline 487 (OECD, 2016). An initial preliminary cytotoxicity test was conducted using 3-FL at concentrations up to 2,000 µg/mL— the OECD Test Guideline 487 maximum recommended concentration—in the presence (3-hour treatment) and absence (3- and 24-hour treatments) of S9 metabolic activation. No precipitate was observed and there were no significant reductions in the cytokinesis-block proliferative index at any 3-FL concentration tested, compared with vehicle controls.

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

There were no biologically relevant or statistically significant differences in the number of binucleate cells containing micronuclei for 3-FL-exposed cultures compared with vehicle controls. Mean micronucleus frequencies for the vehicle control and test item-exposed cultures were all within the laboratory historical 95% CLs. The positive control compounds caused statistically significant increases in the number of binucleate cells containing micronuclei under appropriate conditions (with all mean values falling within the laboratory's historical control ranges), demonstrating the efficacy of the S9 mix and the sensitivity of the

test system. It was concluded, therefore, that 3-FL is neither clastogenic nor aneugenic at concentrations up to 2,000 µg/mL (the OECD Test Guideline 487 maximum recommended concentration).

C.2.2 Clinical Data

5 HMOs (including 3-FL)

Two randomised controlled trials have been conducted in healthy term infants aged ≤ 14 days of age provided either a control formula or a test formula supplemented with 5.75 g/L of a mixture of 5 manufactured HMOs, containing 3-FL in combination with 2'-FL, LNT, 6'-SL and 3'-SL, from enrolment to 4 months of age (Parschat *et al.*, 2021 [NCT03513744]; Lasekan *et al.*, 2022 [NCT04105686]). Both studies also included a reference group of non-randomised breastfed infants. These clinical studies were identified in a recent systematic review of clinical studies on the supplementation of manufactured HMOs (Schönknecht *et al.*, 2023), and have previously been summarised and evaluated by FSANZ as part of Application A1265 (FSANZ, 2023b).

The concentration of individual HMOs provided in the test formula of each study is summarised in Table 9.

Table 9 Concentration of Individual HMOs added to Test Formulas in 5 HMO Trials with 3-FL

HMO	Concentration (g/L) Parschat *et al.*, 2021	Concentration (g/L) Lasekan *et al.*, 2022
2′-FL	2.99	3.0
LNT	1.5	1.5
3-FL	**0.75**	**0.8**
6′-SL	0.28	0.3
3′-SL	0.23	0.2

2′-FL = 2′-fucosyllactose; 3-FL = 3-fucosyllactose; 3′-SL = 3′-silalylactose; 6′-SL = 6′-silalylactose; HiMO = human-identical milk saccharide; LNT = lacto-*N*-tetraose.

In both studies it was concluded that the infant formulas supplemented with the 5 HMOs were safe and well-tolerated.

6 HMOs (including 3-FL) plus 2 Probiotics

The growth, tolerance, and safety of the addition of 6 manufactured HMOs (2′-FL, DFL, **3-FL**, LNT, 3′-SL and 6′-SL) to infant formula in combination with 2 probiotics (*B. lactis* and *B. infantis*) has been evaluated in a randomised controlled trial conducted in healthy term infants aged up to 14 days at enrolment up to 15 months of age (Miranda *et al.*, 2023 [abstract]; Clinical Trial Registry NCT04962594). General study design, product groups, and results from benefit-related outcomes are summarised in Section A.2.2 of this part of the application.

Briefly, enrolled infants were randomised to the CF or EF, and a non-randomised parallel reference group of exclusively BF infants was also enrolled. Formula-fed infants received starter infant formula (0 up to 6 months of age), follow-up formula (6 up to 12 months of age), and growing up milk (12 up to 15 months of age) supplemented with (EF group) or without (CF group) the 6 HMOs plus 2 probiotics. Target amounts of total and individual HMOs in EFs are presented in Table 4 of Section A.2.2.

The primary objective of the trial was to demonstrate safety of a combination of 6 HMOs and 2 probiotics in a starter IF by comparing growth (weight gain velocity in g/day) of infants randomised to EF or CF from enrolment to 4 months of age. Weight gain velocity was assessed from enrolment until 4 months of age according to recommendations from the United States Food and Drug Administration and the American Academy of Pediatrics (AAP, 1988) for clinically testing the safety of infant formula. Secondary safety-related outcomes evaluated at different stages of feeding include gastrointestinal (GI) tolerance, GI symptoms, GI-related behaviours, anthropometric measurements, and adverse events (AEs). The primary objective of the trial was analysed in the full analysis set (FAS)[12] and per protocol set (PP)[13]. Other secondary outcomes were analysed in FAS, except AEs which were analysed in the safety analysis set (SAF)[14].

The number of infants in the FAS and PP is already summarised in Section A.2.2 of this part of the application. Briefly, the FAS included 313 infants (118 in EF, 114 in CF, and 81 in BF) and the PP included 227 infants (84 in EF, 84 in CF, and 59 in BF). The SAF, which consisted of all enrolled formula-fed subjects with documented consumption of at least one feeding of the study formula or breastmilk for the BF group, included 314 total infants (118 in EF, 115 in CF, and 81 in BF). Infants in SAF were classified according to the type of feeding received irrespective of the randomisation assignment, and included one infant excluded from FAS because the assigned study product was not consumed. Based on the total number enrolled (n=318), 81.4% infants (n=259) completed the study through to 4 months of age.

Weight gain velocity from enrolment until 4 months of age in EF was non-inferior to CF in both FAS and PP. At all timepoints, EF and CF had similar weight-for-age, length-for-age, head circumference-for-age, BMI-for-age, and weight-for-length z-scores, with the majority of infants falling within the normal range relative to the World Health Organization (WHO) median z-scores (± 0.5 standard deviations).

[12] All subjects who met the study inclusion and exclusion criteria and who received and consumed their allocated product (for CF and EF subjects) or who consumed breastmilk (for BF subjects).
[13] All subjects in the FAS population who adhered to all protocol requirements without any major protocol deviations influencing the primary endpoint (weight gain velocity) and with complete baseline data and at least one post-randomisation data point.
[14] All randomised infants with documented use of at least one feeding of the study formula.

There were no significant differences between EF and CF groups in GI tolerance, assessed using the overall score of the Infant Gastrointestinal Symptom Questionnaire (IGSQ). Scores by IGSQ domain (stooling, spitting up/vomiting, crying, fussiness, and flatulence) were also very similar between groups across timepoints, except at age 1 month, where EF had a significantly higher stooling domain score compared to BF and CF. According to diary data, EF and CF generally had lower mean stool frequency compared to BF through to 4 months, and stool consistency was generally higher in EF and CF compared to BF until the introduction of solid foods (though mean post-baseline scores indicated favourable stool consistency in all groups). The incidence of difficulty passing stools was generally similar between all three groups, except at 3 months where both EF and CF had significantly lower incidence compared to BF. Sleep scores were generally similar between all groups throughout the study.

The incidence of adverse events (AEs) and serious adverse events was low and comparable between EF and CF. Two AEs categorised as "related" to the study product, both in EF, were concluded to be due to cow's milk protein allergy. The incidence of AEs with "probable" relation to the experimental formula was comparable between EF and CF, and there were no significant differences in severity of AEs or discontinuation due to AE across the three groups.

The study authors concluded that infant formula supplemented with 6 HMOs and the 2 probiotics (*B. lactis* and *B. infantis*) supports healthy, age-appropriate growth through 15 months of age, and is well-tolerated and supports normal stool consistency.

C.3 International Safety Assessments

An overview of safety assessments on Glycom's 3-FL conducted by other authoritative bodies with comparable regulatory processes is provided below.

C.3.1 Australia

Following an application by Glycom, the *Therapeutic Goods (Permissible Ingredients) Determination (No. 5) 2022* was updated on 24 November 2022 to include 3-FL for use in complementary medicines at a maximum recommended daily dose of 2 g in infants and young children aged 0 to 3 years and of 5 g in individuals aged 4 years and older, with Glycom holding exclusivity until 13 December 2024. The latest version is *Therapeutic Goods (Permissible Ingredients) Determination (No. 3) 2024* (TGA, 2024)[15].

C.3.2 EU

In the EU, Glycom's 3-FL is permitted for use in infant formula and follow-on (as defined in Regulation (EU) No 609/2013) at a maximum use level of 1.75 g/L in the final product ready for use, among other food uses.

[15] Federal Register of Legislation - Therapeutic Goods (Permissible Ingredients) Determination (No. 3) 2024

This is following the submission by Glycom of a novel food application pursuant to Regulation (EU) 2015/2283 in 2021 for the use of 3-FL produced by microbial fermentation with a derivative strain of *E. coli* K-12 DH1 (as described herein). At the request of the European Commission, the EFSA NDA Panel adopted a Scientific Opinion on 27 April 2023 (first published on 08 June 2023) in which it was concluded that 3-FL is safe for its intended uses as a novel food ingredient (EFSA, 2023).

Following this Scientific Opinion, Commission Implementing Regulation (EU) 2023/2210[16] was issued authorising the placing on the market of Glycom's 3-FL produced by a derivative strain of *E. coli* K-12 DH1 as a novel food in the EU.

C.3.3 UK

In the UK, Glycom's 3-FL is permitted for use in infant formula and follow-on (as defined in Regulation (EU) No 609/2013) at a maximum use level of 2.0 g/L in the final product ready for use, among other food uses.

This is following a novel food application submitted by Glycom to the UK Food Standards Agency (FSA) and Food Standards Scotland (FSS) in 2021 for the use of 3-FL in the UK market, as a separate authorisation process from the EU exists as of 2021. Upon request of the FSA and FSS, the ACNFP conducted a risk assessment for 3-FL under the EU novel foods legislation [Regulation (EU) 2015/2283] that has been retained in UK law. In the safety assessment finalised on 15 January 2024, the ACNFP concluded that, under the proposed conditions of use, 3-FL is safe, is not considered to be nutritionally disadvantageous, and does not mislead consumers (ACNFP, 2024).

Following this Safety Assessment, regulations authorising the use of 3-FL produced by a derivative strain of *E. coli* K-12 DH1 came into force on 28 June 2024, via Statutory Instruments from England (2024 No. 685[17], Schedules 6 and 10), Wales [2024 No. 741 (W.102)[18], Schedule 6], and Scotland (2024 No. 156[19], Schedule 6).

C.3.4 United States

[16] Commission Implementing Regulation (EU) 2023/2210 of 20 October 2023 authorising the placing on the market of 3-fucosyllactose produced by a derivative strain of *Escherichia coli* K-12 DH1 as a novel food and amending Implementing Regulation (EU) 2017/2470. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=OJ%3AL_202302210.
[17] The Food Additives and Novel Foods (Authorisations and Miscellaneous Amendments) and Food Flavourings (Removal of Authorisations) (England) Regulations 2024. 2024 No. 685. Available at: https://www.legislation.gov.uk/uksi/2024/685/contents/made
[18] The Food Additives and Novel Foods (Authorisations and Miscellaneous Amendments) and Food Flavourings (Removal of Authorisations) (Wales) Regulations 2024. 2024 No. 741 (W. 102). Available at: https://www.legislation.gov.uk/wsi/2024/741/contents/made
[19] The Food Additives and Novel Foods (Authorisations and Miscellaneous Amendments) and Food Flavourings (Removal of Authorisations) (Scotland) Regulations 2024. 2024 No. 156. Available at: https://www.legislation.gov.uk/ssi/2024/156/contents/made

Glycom's 3-FL has been determined to be Generally Recognized as Safe (GRAS) under its intended conditions of use, including term infant formula for infants up to 12 months of age at a maximum use level of 0.75 g/L as consumed. The GRAS status was notified to the U.S. FDA, and a "no questions" response letter from the U.S. FDA was received by Glycom in November 2022 (GRN 1037 – U.S. FDA, 2022).

D. Information on Dietary Intake of the Nutritive Substance

D.1 Proposed Food Use

3-FL is proposed to be added as a nutritive substance to infant formula products (as defined in Standard 2.9.1).

It is intended to be used alone or in combination with other manufactured HMOs already permitted for use in infant formula products in Australia and New Zealand (as per Schedule 26): 2'-FL, LNnT, 2'-FL/DFL, LNT, 6'-SL and 3'-SL.

D.2 Proposed Maximum Levels in Food Groups or Foods

The maximum proposed use of 3-FL is 2.0 g/L, equivalent to 80 mg/100 KJ based on a minimum infant formula and follow-on formula energy content of 2,500 KJ/L (Standard 2.9.1).

The maximum use level of 3-FL is proposed on the basis of providing similar levels of 3-FL to those occurring on average in human milk. The intention is to achieve dietary intake levels of 3-FL from infant formula products that are within natural intake levels of this HMO from human milk.

D.3 Likely Level of Consumption

The estimated dietary intake of 3-FL under the intended conditions of use in Australia and New Zealand is calculated using model diets for infants 3- and 9-months of age, similar to the approach for Applications A1155 and A1265.

Default values applied in the modal diets are summarised in Table 10.

Table 10 Default Values Applied in Modal Diets for Infants 3- and 9-Months of Age to Estimate the Dietary Intake of 3-FL under the Intended Conditions of Use

Parameter	Value	Source
Recommended energy intake	343 (3 months); 330 (9 months)	United Nations University and World Health Organization, 2004
50th percentile body weight	6.4 (3 months); 8.9 (9 months)	WHO, 2006

Minimum energy content of infant formula (as worst-case)	250 KJ/100g	Food Standards Code, Standard 2.9.1 for Infant formula products (2.9.1—9)

3-FL = 3-fucosyllactose.

The maximum proposed use level of 80 mg of 3-FL per 100 KJ of infant formula product was applied to the modal diets. Estimated dietary intakes at the 90th percentile were estimated by doubling the mean consumption amount.

The following assumptions were considered:

- 1 litre of infant or follow-on formula equals 1,050 g;
- Infants aged 3 months obtain 100% of their energy requirements from infant formula; and
- Infants aged 9 months obtain 50% of their energy requirements from follow-on formula.

Resulting dietary intake estimates are summarised in Table 11.

Table 11 Calculation of Estimated Dietary Intakes of 3-FL Under the Intended Conditions of Use in Infant Formula Products for Infants Aged 3 and 9 Months

Age	Mean EDI (mg/kg bw/day)	P90 EDI[a] (mg/kg bw/day)
3 months	261	523
9 months	126	251

3-FL = 3-fucosylactose; bw = body weight; EDI = estimated dietary Intake; P90 = 90th percentile.

[a] The dietary intake at the 90th percentile was calculated by doubling the mean value.

For infants consuming infant formula products for special dietary use, FSANZ (2023b) previously identified that the following parameters can vary:

1. Infant energy and/or fluid requirements depending on the medical condition; and/or
2. Energy content of infant formula products for special dietary uses.

Infants with similar energy requirements and consuming products with similar energy content to those from the model diets are expected to have similar 3-FL intakes, while infants with similar energy requirements but consuming products with a higher energy content are expected to have lower 3-FL intakes. Other scenarios could result in a dietary exposure to 3-FL above estimated dietary intakes described above using the modal diets, though infants consuming infant formula products for special dietary uses are generally under medical and dietetic supervision, and dietary exposure under these scenarios is expected to be short term. Furthermore, the maximum proposed use level of 3-FL applied in the model diets is within the mean level of 3-FL that occurs in human milk, meaning that breastfed infants consuming higher-than-average volumes of breastmilk from mothers with higher-than-average levels of 3-FL are exposed to higher levels of 3-FL without safety concerns.

D.4 Percentage of Food Group to Use Nutritive Substance

In deriving the estimated dietary intakes of 3-FL from its proposed use in infant formula products, the following conservative assumptions were applied to the infant modal diets:

- There is 100% market penetration of infant formula products containing 3-FL at the maximum proposed use level (2.0 g/L);
- The infant formula products offer the minimum infant formula energy content requirement (resulting in the highest consumption volume to meet the recommended energy intake); and
- Infants 3 months of age consumed solely infant formula containing 3-FL, and infants 9 months of age obtain half of their energy requirements from follow-on formula containing 3-FL.

It is unlikely that 3-FL will have 100% market penetration in infant formula products in Australia and New Zealand, given that HMOs are still relatively new (first HMOs gazetted in the Code in 2021), and consumer awareness and understanding of HMOs and their benefits remains limited (see Part 3.3.3, Section G.1).

D.5 Use in Other Countries

Maximum authorised use levels of 3-FL in other countries with similar regulatory processes for equivalent food categories to infant formula products are summarised in Table 12. The maximum proposed use level of 3-FL in infant formula products in Australia and New Zealand (80 mg/100 kJ, equivalent to 2.0 g/L) is the same as that authorised in the UK for infant formula and follow-on formula.

In addition to infant formula products, 3-FL is also permitted for use in other types of foods in these countries, including baby foods and conventional foods (EU, UK and USA), foods for specifical medical purposes (EU and UK), and food supplements (Australia, EU and UK).

Table 12 Summary of Authorised Maximum Use Levels for 3-FL in Infant Formula Products in Other Countries

Jurisdiction	Food Category	Maximum Use Level (g/L)	Reference
UK	Infant formula and follow-on formula as defined in Regulation (EU) No. 609/2013	2.0	The Food Additives and Novel Foods and Food Flavourings Regulations 2024 (England, Scotland and Wales)
EU	Infant formula as defined under Regulation (EU) No 609/2013	1.75	Commission Implementing Regulation (EU) 2017/2470

Jurisdiction	Food Category	Maximum Use Level (g/L)	Reference
	Follow-on formula as defined under Regulation (EU) No 609/2013		
USA	Non-exempt term infant formula for term infants	0.9	GRN 1099

3-FL = 3-fucosyllactose; EU = European Union; UK = United Kingdom; USA = United States of America.

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

Not applicable.

E. Nutritional Impact of a Vitamin or Mineral

Not applicable.

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

3-FL is proposed to be added to infant formula products at similar levels to those found in human milk, alone or in combination with other HMOs, to bring the composition of infant formula closer to human milk, and to provide the same benefits to formula fed infants as breastfed infants receiving these oligosaccharides from human milk. This is in accordance with provisions summarised in Table 2 for optional ingredients in the Codex Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (Codex Alimentarius, 2023a) and Standard for Follow-Up Formula for Older Infants (Codex Alimentarius, 2023b), as well as the Ministerial Policy Guideline on Infant Formula Products principle that "*the composition of breastmilk should be used as a primary reference for determining the composition of infant formula and follow-on formula*" (Australia and New Zealand Food Regulation Ministerial Council, 2011).

Infant formula products that contain 3-FL would be required to meet the compositional requirements that have been already established in Standard 2.9.1, ensuring that the composition of infant formula and follow-on formula is safe, suitable, and supports normal growth and development. No anti-nutritional effects (*i.e.*, reductions in the availability of nutrients) are expected following the consumption of manufactured HMOs (including 3-FL), as demonstrated by infant clinical trials where infant formulas containing HMOs supported age-appropriate growth (see Section C.2.2).

G. Potential Impact on Consumer Understanding and Behaviour

G.1 Consumer Awareness and Understanding

FSANZ (2023a) previously concluded in their Approval Report for Application A1265 that caregiver understanding and behaviour were not expected to be significantly impacted following the approval of Glycom's four previous HMOs (2'-FL/DFL, LNT, 3'-SL sodium salt and 6'-SL sodium salt). This was based on consumer research data on infant formula labelling indicating that caregivers lack knowledge and understanding on the ingredients listed (FSANZ, 2022).

The commercial use of manufactured HMOs in infant formula products remains relatively new in Australia and New Zealand, since gazettal of the first two HMOs (2'-FL and LNnT) into the Code in 2021[20]. Therefore, it is not expected that caregiver awareness and understanding of HMOs (including 3-FL) and their benefits will have had changed considerably.

An additional 6 infant formula products containing HMOs were identified on the Australian or New Zealand market *via* the Mintel Global New Products Database (GNPD), since the 7 products previously identified in Application A1265. These products are summarised in Table 13 below (data available upon request). The 6 new products identified are premium (or platinum) brands. Consumer research data on infant formula labelling indicate that caregivers look for the presence or absence of particular substances or nutrients in the ingredient list, for example between premium brands and standard infant formulas (FSANZ, 2022).

As healthcare professionals become more familiar with HMOs and their role in infant nutrition, it is anticipated that such products may be recommended to mothers/caregivers who are unable to breastfeed or who choose not to breastfeed.

Table 13 Commercialised Infant Formula Products Containing Manufactured HMOs in Australia or New Zealand

Product	Description
	**Alula Advance+ Stage 1 Newborn Premium Infant Formula**, containing 100 mg of 2'-FL and 50 mg of LNnT per 100 mL.

[20] Federal Register of Legislation - Food Standards (Application A1155 – 2'-FL and LNnT in infant formula and other products) Variation.

Product	Description
	**Alula Advance+ Stage 2 Follow-On Premium Infant Formula**, containing 100 mg of 2'-FL and 50 mg of LNnT per 100 mL.
	**Future Gradulac Gentle Stage 1 Newborn Infant Nourish PP+ Premium Formula**, containing 82 mg of 2'-FL per 100 mL.
	**Future Gradulac Gentle Stage 2 Follow-On Nourish PP+ Premium Formula**, containing 82 mg of 2'-FL per 100 mL.
	**Oli6 Stage 1 Goat Milk Infant Formula**, containing 24 mg of 2'-FL and 6 mg of LNnT per 100 mL.
	**Oli6 Stage 2 Goat Follow on Formula**, containing 24 mg of 2'-FL and 6 mg of LNnT per 100 mL.
	**Biostime SN-2 Bio Plus Stage 1 HPO Infant Formula**, containing 0.94 g of 2'-FL per 100 g.

Product	Description
New Products Identified since Application A1265	
	**S-26 Gold Newborn Stage 1 Premium Infant Formula**, containing 194 mg of 2′-FL per 100 g.
	**S-26 Gold Stage 2 Premium Follow-On Formula,** containing 195 mg of 2′-FL per 100 g.
	**NAN SupremePro Stage 1 Premium Starter Infant Formula,** containing 87 mg of 2′-FL, 11 mg of 3′-SL, 14 mg of 6′-SL, 12 mg of DFL and 29 mg of LNT per 100 mL.
	**NAN SupremePro Stage 2 Premium Follow-On Formula,** containing 26 mg of 2′-FL, 6.3 mg of 3′-SL, 4.7 mg of 6′-SL, 3.7 mg of DFL and 8.9 mg of LNT per 100 mL.
	**Oz Farm Super Platinum Stage 2 Follow-On Formula**, containing 400 mg of 2'-FL and 200 mg of LNnT per 100 g.
	**NAN Expert Pro SensiPro Premium Starter Infant Formula,** containing 87 mg of 2′-FL, 11 mg of 3′-SL, 14 mg of 6′-SL, 12 mg of DFL and 29 mg of LNT per 100 mL.
2'FL = 2'-fucosyllactose; 3′-SL = 3′-sialyllactose; 6′-sialyllactose; DFL = difucosyllactose; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose.	

G.2 Actual or Potential Behaviour of Consumers

Caregiver behaviour is not expected to be significantly impacted by the approval of 3-FL in infant formula products, similar to the previous conclusion by FSANZ for other HMOs in Approval Report for Application A1265 (FSANZ, 2023a).

As indicated in Section G.1, commercialization of infant formula products containing HMOs is relatively new, and consumer awareness and understanding of HMOs and their benefits remains limited.

Australia's National Health and Medical Research Council (NHMRC) and New Zealand's Ministry of Health recommend exclusive breastfeeding to around 6 months of age and for breastfeeding to continue alongside the introduction of solid foods until 12 or 24 months of age or longer as desired by the mother and child desire (NHMRC, 2012, Ministry of Health 2021). Infant formula is the only safe and suitable alternative to breastfeeding to meet an infant's primary nutritional needs. As the purpose of adding 3-FL to infant formula products is to make its composition closer to human milk, which is considered the gold standard of infant feeding, it is expected that such products will be well-received by mothers/caregivers who are unable to breastfeed or who choose not to breast feed.

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

The manufactured 3-FL is chemically and structurally identical to 3-FL in human milk (see Appendix 3). The maximum proposed use level of 3-FL in infant formula products is based on 3-FL levels observed in human milk. As a result, the dietary intake of 3-FL by formula-fed infants would be comparable to the dietary intake of 3-FL by breastfed infants.

No safety concerns have been identified for 3-FL in product-specific toxicology studies (Phipps *et al.*, 2022), nor in randomised controlled infant clinical trials (Parschat et al., 2021; Lasekan et al., 2022; Miranda *et al.*, 2023 [abstract]). Furthermore, 3-FL in combination with up to 5 other HMOs has already been commercialised in infant formula products in several markets, from which post-market surveillance data do not indicate any untoward effects. These data are provided in Appendix 9 **(Confidential Commercial Information)**.

As lactose is used as a raw material in the manufacture of 3-FL, which is derived from milk (listed in S9—3), infant formula products containing 3-FL will be labelled in accordance with mandatory declarations requirements set out in Division 3 of Standard 1.2.3 of the Code.

Part 3.5.1 – Foods Produced using Gene Technology

A. Technical Information on the Food Produced using Gene Technology

A.1 Nature and Identity

The 3-FL that is the subject of this application meets the definition of "Food produced using gene technology", as defined in Standard 1.5.2, on the basis that it is considered a "*food which has been derived or developed from an organism which has been modified by gene technology*" (FSANZ, 2018).

Specifically, 3-FL is produced by a derivative of *E. coli* strain K-12, similar to other HMOs manufactured by Glycom that are already authorised in Australia and New Zealand (*i.e.*, 2'-FL, LNnT, a combination of 2'-FL/DFL, LNT, 6'-SL sodium salt and 3'-SL sodium salt – see Schedule 26 of the Code). The platform strain optimised for general oligosaccharide expression for all of Glycom's manufactured HMOs has been modified with the introduction of well-defined, synthesised DNA sequences, to enable the biosynthesis of 3-FL. The 3-FL production strain has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in Braunschweig, Germany. The deposition certificate is provided in Appendix 10 **(Confidential Commercial Information)**.

Notably, during the fermentation process, the production microorganism excretes 3-FL extracellularly into the fermentation medium. At the end of the fermentation process, the production microorganism is removed from the fermentation medium, and 3-FL is isolated through a series of purification steps (see Part 3.3.3, Section B.4). The efficient removal of the production microorganism was confirmed by analyses for residual DNA in final production batches of 3-FL. The absence of residual DNA from the production microorganism was confirmed by three different quantitative polymerase chain reaction (qPCR) methods that target short sub-sequences of inserted genes or a short subsequence of the multicopy operon encoding the 23S ribosomal subunit of *E. coli*. The absence of the production microorganism in the finished 3-FL ingredient is further confirmed by batch testing for bacteria from the *Enterobacteriaceae* family as part of the microbiological specifications (see Part 3.3.3, Section B.5.1).

A.2 History of Use of Host and Donor Organisms

A.2.1 Host Organism

The host organism (*E. coli* K-12) used for the manufacture of all of Glycom's HMOs has previously been described in Applications A1155 (Glycom A/S, 2017) and A1265 (Glycom A/S,

2023). Briefly, *E. coli* K-12 was specifically developed and recognised as a "safety strain" for molecular biological research in the 1970s (Manning *et al.*, 1977; Smith, 1978), and was among the first organisms in history of modern sequencing technologies for which the whole genome sequence became available (Blattner *et al.*, 1997). *E. coli* K-12-derived strains cannot colonise in the human gastrointestinal system, and do not produce protein-type toxins (U.S. EPA, 1997). *E. coli* K-12 derivatives are today among the preferred microorganisms for industrial biotechnology with wide application scope (Chen *et al.*, 2013; Theisen and Liao, 2017).

Although *E. coli* does not qualify for Qualified Presumption of Safety status in the EU, *E. coli* K-12 is considered as a safe and non-pathogenic or toxigenic microorganism widely used for biotechnological applications (ZKBS, 2021; EFSA, 2023).

A.2.2 Donor Organism

The 3-FL production organism contains the gene for α-1,3-fucosyl-transferase from *Helicobacter pylori* that catalyses the transfer of fucose from its activated sugar nucleotide form, guanosine 5'-diphosphate fucose, to the 3-position of lactose, resulting in the formation of 3-FL. Biosynthetic genes for some of Glycom's other HMOs produced using gene technology of microbial origin permitted in Australia and New Zealand were obtained from the same donor organism (*i.e.*, 2'-FL and 2'-FL/DFL: gene for 1,2-fucosyltransferase; LNnT: gene for beta-1,4-galactosyltransferase; and LNT: gene for beta-1,3- galactosyltransferase – see Schedule 26 of the Code).

Notably, the donor gene was not isolated or directly amplified from the donor organism but rather derived from *de novo* DNA synthesis based on defined DNA sequences obtained from bioinformatic databases. Furthermore, the identity and function of the α-1,3-fucosyl-transferase enzyme involved in the biosynthesis of 3-FL is well-characterised. Therefore, the introduction of this gene would not confer toxicogenic nor pathogenic properties to the host organism.

A.3 Nature of Genetic Modification

The 3-FL production strain is a fully genomic strain that does not contain any plasmids or episomal vectors. The chromosomal genetic modifications applied to *E. coli* K-12 which led to Glycom's platform strain have previously been described in Application A1155 (Glycom, 2017).

Briefly, defined DNA sequences from donor microorganisms (such as the gene encoding α-1,3-fucosyl-transferase from *Helicobacter pylori*) were identified using genome databanks, codon-optimised by bioinformatic tools (when needed), extended with appropriate restriction enzyme recognition sequences to allow directed cloning, and then synthesised by DNA synthesis. Genetic cassettes containing the synthesised DNA sequences were integrated *via* homologous recombination into the chromosomal DNA of the production strain. Therefore, no unspecified DNA is expected to be transferred.

The genetic modifications applied to the platform strain to generate the 3-FL production strain were verified by applying whole genome sequencing and colony PCR and targeted sequencing methods. The results obtained from sequencing and PCR were compared to the platform strain and confirmed that the 3-FL production strain contains the applied genetic modifications. The 3-FL production strain is highly stable and reliable, providing high titres of 3-FL. The 3-FL production strain was confirmed to be stable for over 50 generations both in terms of phenotypic performance and genotypic stability. Fermentation performance tests on initial and aged production cell cultures also showed that phenotypic titres and growth rates are stable over time.

The genome of the 3-FL production strain has been assessed *in silico* for the presence of antimicrobial resistance genes according to relevant EFSA guidance (EFSA, 2018; EFSA, 2021). No acquired antimicrobial resistance genes resulting from the genetic modifications were identified. Phenotypic antimicrobial susceptibility testing was also performed on the 3-FL production strain according to the requirements of the above EFSA guidance for phenotypic testing of *Enterobacteriaceae* bacteria. The minimum inhibitory concentration results confirmed the susceptibility of the 3-FL production strain to relevant antimicrobials considered critically or highly important antimicrobials by WHO-AGISAR (2016).

The EFSA NDA Panel has evaluated the genetic modifications applied to the platform strain to obtain the 3-FL production strain and concluded that the production process is sufficiently described and does not raise safety concerns (EFSA, 2023). For completeness, 3-FL production strain data submitted to the EFSA NDA Panel, meeting requirements of the EFSA *Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use* (EFSA, 2011), are provided in Appendix 10 **(Confidential Commercial Information)**.

B. Characterisation and Safety Assessment of New Substances

Amino acid sequences of recombinant proteins from the 3-FL production strain were assessed against a curated database of known and putative allergens using Basic Local Alignment Search Tool (BLAST) search algorithms provided by the Allergen Online tool (Version 21) hosted by the Food Allergen Research and Resource Program (FARRP) of the University of Nebraska (FARRP, 2021). The online tool allows search by three different search algorithms each with its own alert limit for potential allergenicity: (i) Full sequence length (FASTA) comparison with an alert limit greater than 50% sequence similarity to hint for potential allergenic potential; (ii) 80 amino acid sequence segments (sliding window) comparison with an alert limit greater than 35% sequence similarity to hint for potential allergenic potential; and (iii) 8 mer sequence segments (sliding window) with an alert limit of full match to hint for potential allergenic potential. No sequence alerts for potential cross-reactivity to known allergens were identified.

Furthermore, a specification limit for residual proteins (< 0.01 w/w %) has been established for 3-FL (see Part 3.3.3, Section B.5.1), similar to other HMOs produced using gene technology of microbial origin permitted in Australia and New Zealand (see Schedule 3 of the Code).

C. Nutritional Impact of the Food Produced using Gene Technology

Not applicable.

D. Other Information

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

Part 3.6.2 – Special Purpose Food: Infant Formula Products

A. Information Related to Composition

A.1 Purpose of Compositional Change

See Part 3.3.3, Section A.1, of the application.

A.2 Data for Supporting Evidence

See Section A.3 that follows.

A.3 Specific Information Requirements

A.3.1 Characterisation of Proposed Substance in Breast Milk

A.3.1.1 Human Milk Oligosaccharides

As previously discussed in Applications A1155 and A1265, HMOs are a complex family of structurally related oligosaccharides that form the third largest solid component in human milk after lactose and fat (Kuhn, 1952; Kunz and Rudloff, 1993; Bode, 2012; Newburg, 2013). Briefly, more than 140 members of this family have been fully described on a structural basis (Urashima *et al.*, 2011; Chen, 2015), and an even higher number of members have been detected by sensitive mass spectrometry techniques (Finke *et al.*, 1999; Wu *et al.*, 2010, 2011). The highest concentrations of HMOs occur in human colostrum (20 to 25 g/L), and concentrations between 5 to 20 g/L are present in mature human milk (Bode, 2012). Levels of HMOs vary between individuals and are dependent on factors such as the lactation period (levels generally decrease as lactation progresses) and the genotype of the mother (*e.g.*, Lewis or Secretor genes encoding fucosyltransferases) (Castanys-Muñoz *et al.*, 2013). In contrast, bovine colostrum contains approximately 20 times lower concentrations of a far less complex oligosaccharide mixture and concentrations drop to insignificant levels at mature cow milk (Tao *et al.*, 2009; Aldredge *et al.*, 2013; Urashima *et al.*, 2013).

3-FL belongs to the "fucosylated" sub-fraction of HMOs, oligosaccharides that contain the sugar fucose and which is reported to constitute on average around 70 % of the total HMO fraction. In contrast, cow milk does not contain fucosylated oligosaccharides at any appreciable level (Gopal and Gill, 2000; Aldredge *et al.*, 2013). The fucosylated sub-fraction of HMOs is biosynthesised from D-lactose by specific enzymes expressed in mammary glands called fucosyltransferases. The Secretor and Lewis status of the mother (*i.e.,* active or inactive copy of genes expressing fucosyltransfereases), categorising human milk into four different phenotypes, influences the presence and levels of fucosylated HMOs in human milk (reviewed by Castanys-Muñoz *et al.*, 2013). Specifically, the biosynthesis of 3-FL relies on the expression

of α-1,3-fucosyltransferase, which can be encoded by genes that are not subject to populational genetic knock-out polymorphisms. As a result, 3-FL occurs in the milk of all milk groups (phenotypes). Nevertheless, 3-FL is found at highest concentration in the Milk Group 2, which is a *fut2* knock out, but possesses the functional *fut3* gene [encoding a α-(1,3/4)-fucosyltransferase.

What is unique about 3-FL is that it is one of few HMOs observed to increase in concentration over the course of lactation.

A.3.1.2 Levels in Human Breast Milk

EFSA recently outsourced a scoping literature review on mean concentrations of single and total HMOs in human milk (Malih *et al.*, 2024), as part of a request from the European Commission to provide a scientific and technical assistance on the evaluation of manufactured HMOs as novel foods (EFSA, 2024).

The search strategy of the scoping review utilised research databases (PubMed and Web of Science) for studies published between from 01 January 2013 to 16 March 2024, and two systematic reviews (Thurl et al., 2017; Soyyılmaz *et al.*, 2021) to capture studies published before 2013. Primary studies and review studies with full text published in English or Spanish were included, though descriptive statistics were calculated based on primary studies only. The mean of means and maximum mean concentrations of total HMOs, individual HMOs (including 3-FL), and groups of HMOs in human milk is provided by publication or by sample group of donors. For HMO concentrations by sample group of donors, groups of subjects were sorted according to maternal characteristics and lactation phase. These summary statistics were considered by EFSA to better reflect the broad variability of HMOs in human milk and were used by EFSA to derive estimated dietary intakes of HMOs from breast milk for the evaluation (see Section A.3.1.3 that follows). HMO concentrations from colostrum were excluded from summary statistic calculations. As such, EFSA considered summary statistics reported by Malih *et al.* (2024) to be representative of HMO concentration ranges in mature human milk.

Mean concentrations of total HMOs and 3-FL in human milk by sample group of donors reported in the scoping review are summarised in Table 14.

Table 14 Mean Concentration of Total HMOs and 3-FL in Human Milk by Sample Group of Donors, Excluding Colostrum (Reproduced from Malih *et al.*, 2024)

Age	No.	Mean of Means (SD) Concentration (g/L)	Maximum Mean Concentration (g/)
3-FL	275	0.9 (0.7)	5.0
Total HMOs	105	6.7 (5.5)	23.4

3-FL = 3-fucosylactose; HMO = human milk oligosaccharide; SD = standard deviation.

A.3.1.3 Estimated Dietary Intake from Human Breast Milk

The estimated dietary intake of 3-FL and total HMOs from human milk by breastfed infants are calculated using model diets for infants 3- and 9-months of age, similar to the approach for Applications A1155 and A1265.

Default values applied in the modal diets are summarised in Table 15.

Table 15 Default Values Applied in Modal Diets for Infants 3- and 9-Months of Age to Estimate the Dietary Intake of 3-FL from Human Milk

Parameter	Value	Source
Recommended energy intake	343 (3 months); 330 (9 months)	United Nations University and World Health Organization, 2004
50th percentile body weight	6.4 (3 months); 8.9 (9 months)	WHO, 2006
Minimum energy content of infant formula (as worst-case)	286 KJ/100g	AUSNUT 2011-13 food nutrient database

3-FL = 3-fucosyllactose.

Mean concentrations reported in the Malih *et al.* (2024) scoping literature review were applied to the modal diets (see Table 14). The 'mean of means' concentration was applied in the 'mean concentration in human milk' scenario, while the 'maximum mean' concentration was applied in the 'high concentration in human milk' scenario.

The following assumptions were considered:

- 1 litre of human milk equals 1,040 g;
- Infant aged 3 months obtain 100% of their energy requirements from human milk; and
- Infants aged 9 months obtain 50% of their energy requirements from human milk.

Resulting dietary intake estimates are summarised in Table 16.

Table 16 Calculation of Estimated Dietary Intakes of 3-FL and Total HMOs from Human Milk for Infants Aged 3 and 9 Months

HMO	Age	Estimated Dietary Intake (mg/kg bw/day)			
		Mean Concentration in Human Milk		High Concentration in Human Milk	
		Mean	P90	Mean	P90
3-FL	3 months	105	209	577	1,153
	9 months	50	101	277	555
Total HMOs	3 months	777	1,554	2,694	5,387
	9 months	374	748	1,296	2,592

3-FL = 3-fucosylactose; bw = body weight; HMO = human milk oligosaccharide; P90 = 90th percentile.
[a] Dietary intakes at the 90th percentile were calculated by doubling the mean value.

A.3.2 Nutritional Safety and Tolerance

See Part 3.3.3, Section C.2.2.

A.3.3 Efficacy of Proposed Compositional Change

See Part 3.3.3, Section A.2.

B. Dietary Intake or Dietary Exposure

B.1 Dietary Intake or Exposure of Target Population

Table 17 below compares estimated dietary intakes of 3-FL from the maximum intended use level of 2.0 g/L in infant formula products (taken from Table 11) to estimated dietary intakes of 3-FL by breastfed infants from human milk (taken from Table 16).

Anticipated dietary intakes of 3-FL under the intended conditions of use are well-within the anticipated intakes of 3-FL from human milk by breastfed infants.

Table 17 Comparison of 3-FL Estimated Dietary Intakes from the Intended Conditions of Use in Infant Formula Products *versus* Human Milk

Age	Estimated Dietary Intake (mg/kg bw/day)			
	Mean		P90	
	From Intended Use in IFP[a]	From Human Milk[b]	From Intended Use in IFP[a]	From Human Milk[b]
3 months	261	Up to 577	523	Up to 1,153
9 months	126	Up to 277	251	Up to 555

3-FL = 3-fucosyllactose; bw = body weight; IFP = infant formula products; P90 = 90th percentile.
[a] Taken from Table 11.
[b] Taken from Table 16. Based on maximum mean 3-FL levels in human milk reported in the Malih *et al.* (2024) scoping literature review.

As previously mentioned, six other HMOs are already permitted for use in infant formula products in Australia and New Zealand: 2'-FL, LNnT, 2'-FL/DFL, LNT, 3'-SL and 6'-SL. Maximum permitted use levels of these HMOs in infant formula products (Schedule 29) are consistent with levels naturally occuring in human milk, similar to the maximum proposed use level of 3-FL. The maximum amount of total manufactured HMOs potentially added to infant formula products, and percentage of energy density for carbohydrates in infant formula products, was previously calculated by FSANZ as part of the risk assessment for Application A1265 (FSANZ, 2023b). The same approach was applied herein, including 3-FL, as shown in Table 18.

The total amount of manufactured HMOs that could potentially be added to infant formula products is 232 mg/100 kJ. This is well within the mean concentration of total HMOs in human

milk reported in the Malih *et al.* (2024) scoping literature review (maximum of mean of 23.4 g/L, equivalent to 787 mg/100 kJ[21]).

Furthermore, the contribution of manufactured HMOs (including 3-FL) remains minimal at 2.0% relative to total carbohydrate content in infant formula products (36 to 52%).

Table 18 Total Amount of Manufactured HMOs (Including 3-FL) that could be Added to Infant Formula Products, and Contribution to Energy Density (Based on Approach by FSANZ, 2023b)

Manufactured HMO	Maximum Amount (mg/100kJ)	Energy Density (%)
2′-FL or 2′-FL plus LNnT or 2′-FL/DFL	96	0.8
3-FL	80	0.7
LNT	32	0.3
6′-SL	16	0.1
3′-SL	8	0.1
Total	**232**	**2.0**

2′-FL = 2′-fucosyllactose; 3-FL = 3-fucosyllactose; 3′-SL = 3′-silalyllactose; 6′-SL = 6′-sialyllactose; DFL = difucosyllactose; HMO = human milk oligosaccharide; LNnT = lacto-*N*-neotetraose; LNT = lacto-*N*-tetraose.

B.2 Level of Formula Consumption

Not applicable.

B.3 Information Relating to the Substance

3-FL is a natural component of human breast milk. 3-FL is intended to be added to infant formula products at a level that provides formula-fed infants with a similar exposure to 3-FL as breastfed infants. As infant formula is a substitute to breastmilk, exposure to 3-FL is expected to be similar between formula-fed and breastfed infants.

HMOs including 3-FL are also present in other mammalian milks, but not to any significant degree compared to human milk levels (Urashima *et al.*, 2013; Albrecht *et al.*, 2014; Wang *et al.*, 2020). For example, depending on the stage of lactation, human milk can contain HMOs up to 25 g/L in colostrum and up to 20 g/L in mature milk, whereas bovine colostrum contains up to 1 g/L of oligosaccharides that steadily decreases as lactation progresses (Bode, 2012; Urashima *et al.*, 2013). Therefore, there is likely to be minimal contribution from these sources and their by-products to total dietary intakes of 3-FL in infants.

[21] Calculated based human milk having an energy content of 286 KJ/100g, and assuming human milk has a density of 1,040 g/L.

C. Labelling Requirements under Part 2.9 of the Code

C.1 Safety or Nutritional Impact of Labelling Change

See Part 3.3.3, Section B.7.

C.2 Demonstrated Consumer Understanding of Labelling Change

See Part 3.3.3, Section G.1.

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

In addition to the general labelling requirements established under Part 1.2 of the Code, infant formula products containing 3-FL will be labelled in accordance with the specific provisions established in Standard 2.9.1 of the Code.

International labelling standards that are applicable to infant formula products include labelling standards in Article 9 of the International Code of Marketing of Breastmilk Substitutes (WHO, 1981), and the following Codex Alimentarius guidelines and standards:

- Guidelines on Nutrition Labelling (Codex Alimentarius, 2021);
- General Standard for the Labelling of Prepackaged Foods (Codex Alimentarius, 2018);
- General Standard for the Labelling of and Claims for Prepackaged Foods for Special Dietary Uses (Codex Alimentarius, 2009);
- Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (Codex Alimentarius, 2023a); and
- Standard for Follow-up formula for Older Infants and Product for Young Children (Codex Alimentarius, 2023b).

References

AAP (1988). *Clinical Testing of Infant Formulas with Respect to Nutritional Suitability for Term Infants*. (Prepared under FDA contract 223-86-2117). Prepared by Elk Grove Village (IL): American Academy of Pediatrics (AAP) for College Park (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Nutritional Products, Labeling, and Dietary Supplements. Available at: https://web.archive.org/web/20170722090324/http://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/InfantFormula/ucm170649.htm [html, page last updated: 07/09/2015]; https://wayback.archive-it.org/7993/20170405163038/https://www.fda.gov/ohrms/dockets/ac/02/briefing/3903b1_14.pdf [pdf].

ACNFP (2024). *ACNFP Advice on the Safety of 3-Fucosyllactose (3-FL) as a Novel Food*. (Position Paper Reference Number RP1202). London UK: Advisory Committee on Novel Foods and Processes (ACNFP), Food Standards Agency (FSA). Available at: https://acnfp.food.gov.uk/ACNFPAdviceonthesafetyof3-fucosyllactose(3-FL)asaNovelFood.

Albrecht S, Lane JA, Mariño K, Al Busadah KA, Carrington SD, Hickey RM, et al. (2014). A comparative study of free oligosaccharides in the milk of domestic animals. Br J Nutr 111(7):1313-1328. DOI:10.1017/S0007114513003772.

Aldredge DL, Geronimo MR, Hua S, Nwosu CC, Lebrilla CB, Barile D (2013). Annotation and structural elucidation of bovine milk oligosaccharides and determination of novel fucosylated structures. Glycobiology 23(6):664-676. DOI:10.1093/glycob/cwt007.

Austin S, Bénet T (2018). Quantitative determination of non-lactose milk oligosaccharides. Anal Chim Acta 1010:86-96. DOI:10.1016/j.aca.2017.12.036.

Australia and New Zealand Food Regulation Ministerial Council (2011). Food Regulation Standing Committee: Regulation of Infant Formula Products. Available from: https://www.foodregulation.gov.au/resources/publications/policy-guideline-infant-formula-products [Date last updated: 27 September 2023]

Blattner FR, Plunkett G III, Bloch CA, Perna NT, Burland V, Riley M, et al. (1997). The complete genome sequence of *Escherichia coli* K-12. Science 277(5331):1453-1462 [plus supplementary data]. DOI:10.1126/science.277.5331.1453.

Bode L (2012). Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 22(9):1147-1162. DOI:10.1093/glycob/cws074.

Bode L (2013). Human milk oligosaccharides and their beneficial effects. In: Zibadi S, Watson, RR, Preedy VR, editor. *Handbook of Dietary and Nutritional Aspects of Human Breast Milk*. (Human Health Handbooks, Vol. 5). Wageningen, The Netherlands: Wageningen Academic Publishers, pp. 515-531.

Boll EJ, Lopez DV, Terne M, Hessing S, Parschat K, Jensen SR (2024). Human milk oligosaccharides differentially support gut barrier integrity and enhance Th1 and Th17 cell effector responses in vitro. Front Immunol 15:1359499. DOI:10.3389/fimmu.2024.1359499.

Brand-Miller JC, McVeagh P, McNeil Y, Gillard B (1995). Human milk oligosaccharides are not digested and absorbed in the small intestine of young infants. Proc Nutr Soc Austral 19:44.

Brand-Miller JC, McVeagh P, McNeil Y, Messer M (1998). Digestion of human milk oligosaccharides by healthy infants evaluated by the lactulose hydrogen breath test. J Pediatr 133(1):95-98. DOI:10.1016/S0022-3476(98)70185-4.

Bunesova V, Lacroix C, Schwab C (2016). Fucosyllactose and L-fucose utilization of infant *Bifidobacterium longum* and *Bifidobacterium kashiwanohense*. BMC Microbiol 16(1):248 [12pp]. DOI:10.1186/s12866-016-0867-4.

Castanys-Muñoz E, Martin MJ, Prieto PA (2013). 2'-Fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutr Rev 71(12):773-789. DOI:10.1111/nure.12079.

Chaturvedi P, Warren CD, Buescher CR, Pickering LK, Newburg DS (2001). Survival of human milk oligosaccharides in the intestine of infants. In: Newburg DS, editor. *Bioactive Components of Human Milk*. 8th International Conference of the International Society for Research on Human Milk and Lactation, Oct. 25-29, 1997, Plymouth, Mass. (Advances in Experimental Medicine and Biology, vol 501). New York (NY): Kluwer Academic/Plenum Publishers, pp. 315-323.

Chen X (2015). Human Milk Oligosaccharides (HMOS): structure, function, and enzyme-catalyzed synthesis. Adv Carbohydr Chem Biochem 72:113-190. DOI:10.1016/bs.accb.2015.08.002.

Chen X, Zhou L, Tian K, Kumar A, Singh S, Prior BA, et al. (2013). Metabolic engineering of *Escherichia coli*: a sustainable industrial platform for bio-based chemical production. Biotechnol Adv 31(8):1200-1223. DOI:10.1016/j.biotechadv.2013.02.009.

Cheng L, Kiewiet MBG, Logtenberg MJ, Groeneveld A, Nauta A, Schols HA, et al. (2020). Effects of different human milk oligosaccharides on growth of *Bifidobacteria* in monoculture and co-culture with *Faecalibacterium prausnitzii*. Front Microbiol 11:569700 [10pp, plus supplementary figure]. DOI:10.3389/fmicb.2020.569700.

Codex Alimentarius (2009). *General Standard for the Labelling of and Claims for Prepackaged Foods for Special Dietary Uses*. (CXS 146, adopted in 1985. Amendment: 2009). Rome, Italy: Codex Alimentarius Commission. Available at: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B146-1985%252FCXS_146e.pdf.

Codex Alimentarius (2018). *General Standard for the Labelling of Prepackaged Foods*. (CXS 1, adopted in 1985. Amendment: 1991, 1999, 2001, 2003, 2005, 2008 and 2010, Revision: 2018). Rome, Italy: Codex Alimentarius Commission. Available at: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B1-1985%252FCXS_001e.pdf.

Codex Alimentarius (2021). *Guidelines on Nutrition Labelling*. (CXG 2, adopted in 1985. Amendment: 2003, 2006, 2009, 2010, 2012, 2013, 2015, 2016, 2017 and 2021, Revision: 1993 and 2011). Rome, Italy: Codex Alimentarius Commission. Available at: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXG%2B2-1985%252FCXG_002e.pdf.

Codex Alimentarius (2023a). *Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants*. (CXS 72, adopted in 1981. Amendment: 1983, 1985, 1987, 2011, 2015, 2016, 2020 and 2023, Revision: 2007). Rome, Italy: Codex Alimentarius Commission. Available at: fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B72-1981%252FCXS_072e.pdf.

Codex Alimentarius (2023b). *Standard for Follow-Up Formula for Older Infants and Product for Young Children*. (CXS 156, adopted in 1987. Amendment: 1989, 2011 and 2017, Revision: 2023). Rome, Italy: Codex Alimentarius Commission. Available at: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FStandards%252FCXS%2B156-1987%252FCXS_156e.pdf.

Coppa GV, Pierani P, Zampini L, Bruni S, Carloni I, Gabrielli O (2001). Characterization of oligosaccharides in milk and feces of breast-fed infants by high-performance anion-exchange chromatography. In: Newburg DS, editor. *Bioactive Components of Human Milk: 8th International Conference of the International Society for Research on Human Milk and Lactation*, Oct. 25-29, 1997, Plymouth, Mass. (Advances in Experimental Medicine and Biology, Vol. 501). New York (NY): Kluwer Academic/Plenum Publishers, pp. 307-314.

Coppa GV, Zampini L, Galeazzi T, Facinelli B, Ferrante L, Capretti R, et al. (2006). Human milk oligosaccharides inhibit the adhesion to Caco-2 cells of diarrheal pathogens: *Escherichia coli*, *Vibrio cholerae*, and *Salmonella fyris*. Pediatr Res 59(3):377-382. DOI:10.1203/01.pdr.0000200805.45593.17.

De Leoz ML, Wu S, Strum JS, Niñonuevo MR, Gaerlan SC, Mirmiran M, et al. (2013). A quantitative and comprehensive method to analyze human milk oligosaccharide structures in the urine and feces of infants. Anal Bioanal Chem 405(12):4089-4105. DOI:10.1007/s00216-013-6817-1.

Derya SM, Spiegel H, Hanisch FG, Morozov V, Schroten H, Jennewein S, et al. (2020). Biotechnologically produced fucosylated oligosaccharides inhibit the binding of human noroviruses to their natural receptors. J Biotechnol 318:31-38. DOI:10.1016/j.jbiotec.2020.05.001.

Dotz V, Rudloff S, Blank D, Lochnit G, Geyer R, Kunz C (2014). ^{13}C-labeled oligosaccharides in breastfed infants' urine: individual-, structure-and time-dependent differences in the excretion. Glycobiology 24(2):185-194. DOI:10.1093/glycob/cwt099.

Dotz V, Rudloff S, Meyer C, Lochnit G, Kunz C (2015). Metabolic fate of neutral human milk oligosaccharides in exclusively breast-fed infants. Mol Nutr Food Res 59(2):355-364. DOI:10.1002/mnfr.201400160.

Du Z, Li Z, Guang C, Zhu Y, Mu W (2024). Recent advances of 3-fucosyllactose in health effects and production. Arch Microbiol 206(9):378. DOI:10.1007/s00203-024-04104-2.

Dupin L, Noël M, Bonnet S, Meyer A, Géhin T, Bastide L, et al. (2018). Screening of a Library of Oligosaccharides Targeting Lectin LecB of Pseudomonas Aeruginosa and Synthesis of High Affinity Oligoglycoclusters. Molecules 23(12):3073. DOI :10.3390/molecules23123073.

EFSA (2011). Scientific opinion: Guidance on the risk assessment of genetically modified microorganisms and their products intended for food and feed use (EFSA Panel on Genetically Modified Organisms/GMO) (Question no: EFSA-Q-2009-00521, adopted: 25 May 2009 by European Food Safety Authority). EFSA J 9(6):2193 [54pp]. DOI:10.2903/j.efsa.2011.2193. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/2193.

EFSA (2014). Scientific Opinion on the essential composition of infant and follow-on formulae (EFSA Panel on Dietetic Products, Nutrition and Allergies/NDA) (Question no: EFSA-Q-2012-01009, adopted: 20 June 2014 by European Food Safety Authority). EFSA J 12(7):3760 [106pp]. DOI:10.2903/j.efsa.2014.3760. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/3760.

EFSA (2017). Guidance on the risk assessment of substances present in food intended for infants below 16 weeks of age (EFSA Scientific Committee) (Question no: EFSA-Q-2016-00489, adopted: 26 April 2017 by European Food Safety Authority). EFSA J 15(5):4849 [58pp]. DOI:10.2903/j.efsa.2017.4849. Available at: https://www.efsa.europa.eu/en/efsajournal/pub/4849.

EFSA (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms (EFSA Panel on Additives and Products or Substances Used in Animal Feed/FEEDAP) (Question nos: EFSA-Q-2016-00069, EFSA-Q-2017-00211, adopted: 21 February 2018 by European Food Safety Authority). EFSA J 16(3):5206 [24pp].
DOI:10.2903/j.efsa.2018.5206. Available at:
https://www.efsa.europa.eu/en/efsajournal/pub/5206.

EFSA (2021). EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain (Question no: EFSA-Q-2019-00434, adopted: 2 March 2021, by European Food Safety Authority). EFSA J 19(7):6506 [14pp]. DOI:10.2903/j.efsa.2021.6506. Available at:
https://www.efsa.europa.eu/en/efsajournal/pub/6506.

EFSA (2023). Scientific Opinion on the safety of 3-fucosyllactose (3-FL) produced by a derivative strain of *Escherichia coli* K-12 DH1 as a novel food pursuant to Regulation (EU) 2015/2283. (EFSA Panel on Nutrition, Novel Foods and Food Allergens/NDA) (Question no: EFSA-Q-2021-00354, adopted: 27 April 2023 by European Food Safety Authority). EFSA J 21(6):8026 [29pp]. DOI:10.2093/j.efsa.2023.8026. Available at:
https://www.efsa.europa.eu/en/efsajournal/pub/8026.

EFSA (2024). Scientific and technical assistance report on the evaluation of human-identical milk oligosaccharides (HiMOs) as novel foods (Question no: EFSA-Q-2024-00049, approved: 26 August 2024 by European Food Safety Authority). EFSA Supporting publication 2024:EN-8994 [33pp]. DOI:10.2903/sp.efsa.2024.EN-8994. Available at:
https://www.efsa.europa.eu/en/supporting/pub/en-8994.

Eichele DD, Kharbanda KK (2017). Dextran sodium sulfate colitis murine model: An indispensable tool for advancing our understanding of inflammatory bowel diseases pathogenesis. World J Gastroenterol 23(33):6016-6029. DOI:10.3748/wjg.v23.i33.6016.

Engels C, Ruscheweyh HJ, Beerenwinkel N, Lacroix C, Schwab C (2016). The Common Gut Microbe Eubacterium hallii also Contributes to Intestinal Propionate Formation. Front Microbiol 7:713. DOI:10.3389/fmicb.2016.00713.

Engfer MB, Stahl B, Finke B, Sawatzki G, Daniel H (2000). Human milk oligosaccharides are resistant to enzymatic hydrolysis in the upper gastrointestinal tract. Am J Clin Nutr 71(6):1589-1596. DOI:10.1093/ajcn/71.6.1589.

EU (2023). Commission implementing Regulation (EU) 2023/2210 of 20 October 2023 authorising the placing on the market of 3-Fucosyllactose produced by a derivative strain of *Escherichia coli* K-12 DH1 as a novel food and amending Implementing Regulation (EU) 2017/2470. Off J Eur Union. Available at: https://eur-lex.europa.eu/eli/reg_impl/2023/2210/oj.

FARRP (2021). *AllergenOnline Version 21: Home of the FARRP Allergen Protein Database*. Lincoln (NE): University of Nebraska-Lincoln, Food Allergy Research and Resource Program (FARRP). Available at: http://www.allergenonline.org/ [Released: February 14, 2021].

Finke B, Stahl B, Pfenninger A, Karas M, Daniel H, Sawatzki G (1999). Analysis of high-molecular-weight oligosaccharides from human milk by liquid chromatography and MALDI-MS. Anal Chem 71(17):3755-3762. DOI:10.1021/ac990094z.

FSANZ (2018). Standard 1.5.2 – Food produced using gene technology. (F2018C00169). In: *Australia New Zealand Food Standards Code*. Canberra, Australia: Food Standards Australia New Zealand (FSANZ), Australian Government. Available at: https://www.legislation.gov.au/Details/F2018C00169.

FSANZ (2019). *Approval Report – Application A1155: 2'-FL and LNnT in Infant Formula and Other Products*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/A1155%20Approval%20Report%20for%20web.pdf

FSANZ (2020). *Review – Application A1155: 2'-FL and LNnT in Infant Formula and Other Products*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/A1155%20Review%20Report.pdf.

FSANZ (2022). *Proposal P1028 – Infant formula – Attachment 1 – Consumer research on infant formula labelling*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/proposals/Documents/Attachment%201%20to%20SD3%20-%20Consumer%20research%20on%20infant%20formula%20labelling.pdf.

FSANZ (2023a). *Approval Report – Application A1265. A1265 – 2'-FL/DFL, LNT, 6'-SL sodium salt and 3'-SL sodium salt as nutritive substances in infant formula products*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/sites/default/files/2023-11/A1265%20Approval%20Report.pdf.

FSANZ (2023b). *Supporting Document 1 (at approval). Risk Assessment – Risk, technical and benefit assessment. Application A1265: 2′-FL/DFL, LNT, 6′-SL sodium salt and 3′-SL sodium salt for use as nutritive substances in infant formula products*. Canberra, Australia / Wellington, New Zealand: Food Standards Australia New Zealand (FSANZ). Available at: https://www.foodstandards.gov.au/sites/default/files/2023-11/A1265%20SD1%20at%20approval.pdf.

Garrido D, Ruiz-Moyano S, Lemay DG, Sela DA, German JB, Mills DA (2015). Comparative transcriptomics reveals key differences in the response to milk oligosaccharides of infant gut-associated bifidobacteria. Sci Rep 5:13517 [18pp & erratum, 5:13511]. DOI:10.1038/srep13517.

Gilby B (2021a) [unpublished]. *3-Fucosyllactose (3-FL): Bacterial Reverse Mutation Test. Final Report: Confidential and Proprietary*. (Covance Study No. 8429603; Feb. 17, 2021). Prepared by Huntingdon, UK: Covance Laboratories Limited for Glycom A/S.

Gilby B (2021b) [unpublished]. *3-Fucosyllactose (3-FL): In Vitro Micronucleus Test in Human Lymphocytes. Final Report: Confidential and Proprietary*. (Covance Study No. 8436271; Feb. 17, 2021). Prepared by Huntingdon, UK: Covance Laboratories Limited for Glycom A/S.

Glycom A/S (2017). *Application for the Approval of 2′-O-Fucosyllactose (2′-FL) and Lacto-N-neotetraose (LNnT) Under Standard 1.5.1 (Novel Foods) of the Australia and New Zealand Food Standards Code*. Prepared Glycom A/S (Hørsholm, Denmark). Submitted to Food Standards Australia New Zealand (Kingston, Australia; Wellington, New Zealand). Available at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/A1155%20Application%20final_Redacted.pdf.

Glycom A/S (2023). *Application to Amend the Australia New Zealand Food Standards Code: 2′-FL/DFL, LNT, 6′-SL Sodium Salt, and 3′-SL Sodium Salt Produced using Gene Technology for Use as Nutritive Substances in Infant Formula Products*. Prepared by Glycom A/S (Hørsholm, Denmark). Submitted to Food Standards Australia New Zealand (Kingston, Australia; Wellington, New Zealand). Available at: https://www.foodstandards.gov.au/sites/default/files/food-standards-code/applications/Documents/Amended%20Application%20to%20FSANZ%20-%202FLDFL,%20LNT,%206SL%20and%203SL%20-%20Amended%2022Mar%2723_Redacted.pdf.

Gnoth MJ, Kunz C, Kinne-Saffran E, Rudloff S (2000). Human milk oligosaccharides are minimally digested in vitro. J Nutr 130(12):3014-3020. DOI:10.1093/jn/130.12.3014.

Goehring KC, Kennedy AD, Prieto PA, Buck RH (2014). Direct evidence for the presence of human milk oligosaccharides in the circulation of breastfed infants. PLoS ONE 9(7):e101692 [11pp]. DOI:101610.101371/journal.pone.0101692.

Gopal PK, Gill HS (2000). Oligosaccharides and glycoconjugates in bovine milk and colostrum. Br J Nutr 84(Suppl. 1):S69-S74. DOI:10.1017/S0007114500002270.

Hester SN, Hustead DS, Mackey AD, Singhal A, Marriage BJ (2012). Is the macronutrient intake of formula-fed infants greater than breast-fed infants in early infancy? J Nutr Metab 2012:Article ID 891201 [13pp]. DOI:10.1155/2012/891201.

Holst AQ, Jois H, Laursen MF, Sommer MOA, Licht TR, Bahl MI (2022). Human milk oligosaccharides induce acute yet reversible compositional changes in the gut microbiota of conventional mice linked to a reduction of butyrate levels. Microlife 3:uqac006. DOI:10.1093/femsml/uqac006.

Holst AQ, Myers P, Rodríguez-García P, Hermes GDA, Melsaether C, Baker A, et al. (2023). Infant Formula Supplemented with Five Human Milk Oligosaccharides Shifts the Fecal Microbiome of Formula-Fed Infants Closer to That of Breastfed Infants. Nutrients 15(14):3087. DOI:10.3390/nu15143087.

ICH (2020). *Nonclinical Safety Testing in Support of Development of Paediatric Pharmaceuticals: S11.* (ICH Harmonised Guideline – Final Version, Adopted on 14 April 2020). Geneva, Switzerland: International Council for Harmonisation of Technical Requirements for Pharmaceutical for Human Use (ICH). Available at: https://database.ich.org/sites/default/files/S11_Step4_FinalGuideline_2020_0310.pdf.

Kim YJ, Kim HH, Shin CS, Yoon JW, Jeon SM, Song YH, et al. (2023). 2'-Fucosyllactose and 3-Fucosyllactose Alleviates Interleukin-6-Induced Barrier Dysfunction and Dextran Sodium Sulfate-Induced Colitis by Improving Intestinal Barrier Function and Modulating the Intestinal Microbiome. Nutrients 15(8):1845. DOI:10.3390/nu15081845.

Kong C, Akkerman R, Klostermann CE, Beukema M, Oerlemans MMP, Schols HA, et al. (2021). Distinct fermentation of human milk oligosaccharides 3-FL and LNT2 and GOS/inulin by infant gut microbiota and impact on adhesion of *Lactobacillus plantarum* WCFS1 to gut epithelial cells. Food Funct 12(24):12513-12525. DOI:10.1039/d1fo02563e.

Kuhn R (1952). Vitamine der Milch. [Vitamins in milk]. Angew Chem 64(18):493-500 [German]. DOI:10.1002/ange.19520641802.

Kunz C, Rudloff S (1993). Biological functions of oligosaccharides in human milk. Acta Paediatr 82(11):903-912. DOI:10.1111/j.1651-2227.1993.tb12597.x.

Kunz C, Rudloff S (2017). Compositional analysis and metabolism of human milk oligosaccharides in infants. In: Isolauri E, Sherman PM, Walker WA, editors. *Intestinal Microbiome: Functional Aspects in Health and Disease*. 88th Nestlé Nutrition Institute Workshop, Sep. 22-25, 2016, Playa del Carmens, Mexico. (Nestlé Nutrition Institute Workshop Series, Vol. 88). Vevey, Switzerland: Nestec Ltd. / Basel, Switzerland: S. Karger AG, pp. 137-147. DOI:10.1159/000455398.

Kunz C, Rudloff S, Baier W, Klein N, Strobel S (2000). Oligosaccharides in human milk: structural, functional, and metabolic aspects. Annu Rev Nutr 20:699-722. DOI:10.1146/annurev.nutr.20.1.699.

Lasekan J, Choe Y, Dvoretskiy S, Devitt A, Zhang S, Mackey A, et al. (2022). Growth and gastrointestinal tolerance in healthy term infants fed milk-based infant formula supplemented with five human milk oligosaccharides (HMOs): A randomized multicenter trial. Nutrients 14(13):2625 [19pp]. DOI:10.3390/nu14132625.

Li Z, Zhu Y, Ni D, Zhang W, Mu W (2022). Occurrence, functional properties, and preparation of 3-fucosyllactose, one of the smallest human milk oligosaccharides. Crit Rev Food Sci Nutr 63(28):9364-9378. DOI:10.1080/10408398.2022.2064813.

Lönnerdal B, Erdmann P, Thakkar SK, Sauser J, Destaillats F (2017). Longitudinal evolution of true protein, amino acids and bioactive proteins in breast milk: a developmental perspective. J Nutr Biochem 41:1-11 [plus supplementary figures]. DOI:10.1016/j.jnutbio.2016.06.001.

Malih N, Konieczna J, Fernandez MN, Fiol-deRoque MA, Zamanillo-Campos R, Ricci-Cabello I (2024). External Scientific Report: Preparatory work for the safety evaluation by EFSA of Human-identical Milk Oligosaccharides as Novel Foods (Question no: EFSA-Q-2024-00421, approved: 7 July 2024 by European Food Safety Authority). EFSA Supporting publication 2024:EN-8955 [49pp]. DOI: 10.2903/sp.efsa.2024.EN-8955. Available at: https://www.efsa.europa.eu/en/supporting/pub/en-8955.

Manning PA, Pugsley AP, Reeves P (1977). Defective growth functions in mutants of *Escherichia coli* K12 lacking a major outer membrane protein. J Mol Biol 116(2):285-300. DOI:10.1016/0022-2836(77)90217-0.

McNaught AD (1996). Nomenclature of carbohydrates (IUPAC Recommendations 1996). Pure Appl Chem 68(10):1919-2008. DOI:10.1351/pac199668101919.

Michaelsen KF, Larsen PS, Thomsen BL, Samuelson G (1994). The Copenhagen Cohort Study on Infant Nutrition and Growth: breast-milk intake, human milk macronutrient content, and influencing factors. Am J Clin Nutr 59(3):600-611. DOI:10.1093/ajcn/59.3.600.

Ministry of Health (2021). Healthy Eating Guidelines for New Zealand Babies and Toddlers (0–2 years old). Wellington: Ministry of Health. Available at: https://www.health.govt.nz/publications/healthy-eating-guidelines-for-new-zealand-babies-and-toddlers-0-2-years-old.

Miranda J, Claris O, Gil-Campos M, Salamanca I, Cornette L, Alliet P et al. (2024). Biomarkers of gut maturity, inflammation, and metabolic environment in infants fed formula containing six human milk oligosaccharides, B. infantis LMG11588, and B. lactis CNCM I-3446 *[abstract]*. In: Abstracts of the 56th ESPGHAN Annual Meeting; Milan (Italy); JPGN Reports 5:S1-S1437, 2024. DOI:10.1002/jpr3.12073.

Miranda J, Claris O, Gil-Campos M, Salamanca I, Cornette L, Alliet P, et al. (2023). Infant formula with a specific age-adapted synbiotic blend of six human milk oligosaccharides and B. infantis LMG11588 plus B. lactis CNCM I-3446 supports healthy, age-appropriate growth and is well-tolerated: a staged analysis of a double-blind, randomized, controlled trial *[abstract]*. In: Abstracts of the 15th Excellence in Pediatrics; Paris (France); Cogent Public Health 10:1, 2023. DOI:10.1080/27707571.2023.2275942

Moon S, Lee KW, Park M, Moon J, Park SH, Kim S, et al. (2024). 3-Fucosyllactose-mediated modulation of immune response against virus infection. Int J Antimicrob Agents 64(1):107187. DOI:10.1016/j.ijantimicag.2024.107187.

Newburg DS (2013). Glycobiology of human milk. Biochemistry (Mosc) 78(7):771-785 [Russian edition "Biokhimiya 778(777) 990-1007"].

Newburg DS, Neubauer SH (1995). Carbohydrates in milks: analysis, quantities, and significance. In: Jensen RG, editor. *Handbook of Milk Composition*. San Diego (CA): Academic Press, pp. 273-349.

NHMRC (2012). *Eat for Health. Infant Feeding Guidelines. Information for Health Workers*. (NHMRC Publication Reference: N56). Canberra, Australia: Australian Government, National Health and Medical Research Council (NHMRC) & Department of Health and Aging. Available at: https://www.nhmrc.gov.au/about-us/publications/infant-feeding-guidelines-information-health-workers.

Ninonuevo MR, Park Y, Yin H, Zhang J, Ward RE, Clowers BH, et al. (2006). A strategy for annotating the human milk glycome. J Agric Food Chem 54(20):7471-7480. DOI:10.1021/jf0615810.

Noll AJ, Yu Y, Lasanajak Y, Duska-McEwen G, Buck RH, Smith DF, et al. (2024). Human DC-SIGN binds specific human milk glycans. Biochem J 473(10):1343-53. DOI:10.1042/BCJ20160046.

Noti M, Picaud JC, Vidal K, Moll JM, Miranda J, Claris O, et al. (2024). An age-adapted synbiotic blend of human milk oligosaccharides and B. infantis LMG11588 plus B. lactis may contribute to immune cell homeostasis in formula-fed infants *[abstract]*. In: Abstracts of the 56th ESPGHAN Annual Meeting; Milan (Italy); JPGN Reports 5:S1-S1437, 2024. DOI:10.1002/jpr3.12073.

Obermeier S, Rudloff S, Pohlentz G, Lentze MJ, Kunz C (1999). Secretion of ^{13}C -labelled oligosaccharides into human milk and infant's urine after an oral ^{13}C galactose load. Isotopes Environ Health Stud 35(1/2):119-125. DOI:10.1080/10256019908234084.

OECD (1997). Bacterial reverse mutation test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 471) [adopted: 21 July 1997]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://doi.org/10.1787/9789264071247-en [latest corrected version: 29 June 2020].

OECD (1998). *OECD Principles of Good Laboratory Practice*. (Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1 [ENV/MC/CHEM(98)17]). Paris, France: Organisation for Economic Co-Operation & Development (OECD), Environment Directorate, Chemicals Group and Management Committee, OECD Environmental Health and Safety Publications. Available at: https://www.oecd-ilibrary.org/environment/oecd-principles-on-good-laboratory-practice_9789264078536-en [as revised in 1997].

OECD (2016). *In Vitro* mammalian cell micronucleus test. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 487) [adopted: 19 July 2016]. Paris, France: Organisation for Economic Co-operation and Development (OECD). Available at: https://doi.org/10.1787/9789264264861-en [latest corrected version: 4 July 2023].

OECD (2018). Repeated dose 90-day oral toxicity study in rodents. In: *OECD Guidelines for the Testing of Chemicals*. (OECD Guideline, No. 408) [adopted: 25 June 2018]. Paris, France: Organisation for Economic Co-operation and Development (OECD). DOI:10.1787/9789264070707-en. Available at: https://doi.org/10.1787/9789264070707-en.

Parschat K, Melsaether C, Jäpelt KR, Jennewein S (2021). Clinical evaluation of 16-week supplementation with 5HMO-mix in healthy-term human infants to determine tolerability, safety, and effect on growth. Nutrients 13(7):2871 [19pp, plus supplementary data]. DOI:10.3390/nu13082871.

Phipps KR, Lozon D, Stannard DR, Gilby B, Baldwin N, Mikš MH, et al. (2022). Neonatal subchronic toxicity and in vitro genotoxicity studies of the human-identical milk oligosaccharide 3-fucosyllactose. J Appl Toxicol 42(10):1671-1687. DOI:10.1002/jat.4335.

Picaud JC, Moll JM, Miranda J, Claris O, Gil-Campos M, Salamanca I, et al. (2023). Gut microbiota and probiotic establishment in healthy term infants fed an infant formula with a specific age-adapted synbiotic blend of six human milk oligosaccharides and B. infantis LMG11588 plus B. lactis CNCM I-3446: a staged analysis of a double-blind, randomized, controlled trial *[abstract]*. In: Abstracts of the 15th Excellence in Pediatrics; Paris (France); Cogent Public Health 10:1, 2023. DOI:10.1080/27707571.2023.2275942.

Picaud JC, Moll JM, Miranda-Mallea J, Claris O, Palomino Fernández L, Salamanca de la Cueva I, et al. (2024). Gut microbiota establishment in infants fed formulas with an age-adapted synbiotic blend of six human milk oligosaccharides, B. infantis LMG11588, and B. lactis CNCM I-3446: a staged analysis of a double-blind, randomized trial *[abstract]*. In: Abstracts of the 2024 American Academy of Pediatrics National Conference & Exhibition; Orlando, Florida (USA); Available at: https://aapexperience24.eventscribe.net/

Rudloff S, Pohlentz O, Diekmann L, Egge H, Kunz C (1996). Urinary excretion of lactose and oligosaccharides in preterm infants fed human milk or infant formula. Acta Paediatr 85(5):598-603. DOI:10.1111/j.1651-2227.1996.tb14095.x.

Rudloff S, Obermeier S, Borsch C, Pohlentz G, Hartmann R, Brosicke H, et al. (2006). Incorporation of orally applied ^{13}C-galactose into milk lactose and oligosaccharides. Glycobiology 16(6):477-487. DOI:10.1093/glycob/cwj092.

Rudloff S, Kunz C (2012). Milk oligosaccharides and metabolism in infants. Adv Nutr 3(3, Suppl.):398S-405S. DOI:10.3945/an.111.001594.

Rudloff S, Pohlentz G, Borsch C, Lentze MJ, Kunz C (2012). Urinary excretion of *in vivo* ^{13}C-labelled milk oligosaccharides in breastfed infants. Br J Nutr 107(7):957-963. DOI:10.1017/S0007114511004016.

Salli K, Hirvonen J, Siitonen J, Ahonen I, Anglenius H, Maukonen J (2021). Selective utilization of the human milk oligosaccharides 2'-fucosyllactose, 3-fucosyllactose, and difucosyllactose by various probiotic and pathogenic bacteria. J Agric Food Chem 69(1):170-182 [plus supplementary data]. DOI:10.1021/acs.jafc.0c06041.

Salli K, Hirvonen J, Anglenius H, Hibberd AA, Ahonen I, Saarinen MT, et al. (2023). The Effect of Human Milk Oligosaccharides and *Bifidobacterium longum* subspecies *infantis* Bi-26 on Simulated Infant Gut Microbiome and Metabolites. Microorganisms 11(6):1553. DOI:10.3390/microorganisms11061553.

Schönknecht YB, Moreno Tovar MV, Jensen SR, Parschat K (2023). Clinical Studies on the Supplementation of Manufactured Human Milk Oligosaccharides: A Systematic Review. Nutrients 15(16):3622. DOI: 10.3390/nu15163622.

Smith HW (1978). Is it safe to use *Escherichia coli* K12 in recombinant DNA experiments? J Infect Dis 137(5):655-660. DOI:10.1093/infdis/137.5.655.

Soyyılmaz B, Mikš MH, Röhrig CH, Matwiejuk M, Meszaros-Matwiejuk A, Vigsnæs LK (2021). The mean of milk: a review of human milk oligosaccharide concentrations throughout lactation. Nutrients 13(8):2737 [22pp, plus supplementary tables]. DOI:10.3390/nu13082737.

Stannard D (2021a) [unpublished]. *3-Fucosyllactose (3-FL): 14-Day Dose Range Finding Study in the Neonatal Crl:CD(SD) Rat by Oral (Gavage) Administration. Final Report: Confidential and Proprietary*. (Covance Study No. 8438008; Feb. 16, 2021). Prepared by Eye, UK: Covance Laboratories Limited for Horsholm, Denmark: Glycom A/S.

Stannard D (2021b) [unpublished*]. 3-Fucosyllactose (3-FL): 90-Day Toxicity Study in the Neonatal Crl:CD(SD) Rat by Oral (Gavage) Administration Followed by a 4-Week Recovery Period. Final Study Plan: Confidential and Proprietary*. (Covance Study No. 8437221; July 30, 2021). Prepared by Eye, UK: Covance Laboratories Limited for Horsholm, Denmark: Glycom A/S.

Tao N, Depeters EJ, German JB, Grimm R, Lebrilla CB (2009). Variations in bovine milk oligosaccharides during early and middle lactation stages analyzed by high-performance liquid chromatography-chip/mass spectrometry. J Dairy Sci 92(7):2991-3001. DOI:10.3168/jds.2008-1642.

Theisen M, Liao JC (2017). 5. Industrial biotechnology: Escherichia coli as a host. In: Wittmann C, Liao JC, editors. *Industrial Biotechnology: Microorganisms: Volume 1*. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co. KGaA, pp. 151-181.

Thongaram T, Hoeflinger JL, Chow J, Miller MJ (2017). Human milk oligosaccharide consumption by probiotic and human-associated bifidobacteria and lactobacilli. J Dairy Sci 100(10):7825-7833. DOI:10.3168/jds.2017-12753.

Thurl S, Munzert M, Boehm G, Matthews C, Stahl B (2017). Systematic review of the concentrations of oligosaccharides in human milk. Nutr Rev 75(11):920-933. DOI:10.1093/nutrit/nux044.

UK Government (2024a). *The Novel Food Additives and Novel Foods (Authorisations and Miscellaneous Amendments) and Food Flavourings (Removal of Authorisations) (England) Regulations 2024*. (UK Statutory Instruments, No. 685). Available at: https://www.legislation.gov.uk/uksi/2024/685/contents/made.

UK Government (2024b). *The Novel Food Additives and Novel Foods (Authorisations and Miscellaneous Amendments) and Food Flavourings (Removal of Authorisations) (Wales) Regulations 2024*. UK Statutory Instruments, No. 741 (W. 102). Available at: https://www.legislation.gov.uk/wsi/2024/741/contents/made.

UK Government (2024c). *The Novel Food Additives and Novel Foods (Authorisations and Miscellaneous Amendments) and Food Flavourings (Removal of Authorisations) (Scotland) Regulations 2024*. UK Statutory Instruments, No. 156. Available at: https://www.legislation.gov.uk/ssi/2024/156/contents/made.

U.S. EPA (1997). *Escherichia Coli K-12 Final Risk Assessment: Attachment I--Final Risk Assessment of Escherichia Coli K-12 Derivatives*. Washington (DC): U.S. Environmental Protection Agency (U.S. EPA), Biotechnology Program under the Toxic Substances Control Act (TSCA). Available at: https://www.epa.gov/sites/production/files/2015-09/documents/fra004.pdf [Last updated on September 27, 2012].

U.S. FDA (2022). *Agency Response Letter GRAS Notice No. GRN 1037 [3-Fucosyllactose, Hørsholm, Denmark: Glycom A/S]*. Silver Spring (MD): U.S. Food and Drug Administration (U.S. FDA), Center for Food Safety and Applied Nutrition (CFSAN), Office of Food Additive Safety. Available at: https://www.cfsanappsexternal.fda.gov/scripts/fdcc/index.cfm?set=GRASNotices&id=1037 [Nov. 7, 2022 - FDA response - no questions].

Urashima T, Fukuda K, Kitaoka M, Ohnishi M, Terabayashi T, Kobata A (2011). *Milk Oligosaccharides*. (Nutrition and Diet Research Progress). New York (NY): Nova Biomedical Books.

Urashima T, Asakuma S, Leo F, Fukuda K, Messer M, Oftedal OT (2012). The predominance of type I oligosaccharides is a feature specific to human breast milk. Adv Nutr 3(3):473S-482S. DOI:10.3945/an.111.001412.

Urashima T, Taufik E, Fukuda K, Asakuma S (2013). Recent advances in studies on milk oligosaccharides of cows and other domestic farm animals. Biosci Biotechnol Biochem 77(3):455-466. DOI:10.1271/bbb.120810.

Viverge D, Grimmonprez L, Cassanas G, Bardet L, Solere M (1990). Variations in oligosaccharides and lactose in human milk during the first week of lactation. J Pediatr Gastroenterol Nutr 11(3):361-364. DOI:10.1097/00005176-199010000-00013.

Wang Y, Zhou X, Gong P, Chen Y, Feng Z, Liu P, et al. (2020). Comparative major oligosaccharides and lactose between Chinese human and animal milk. Int Dairy J Article 108, 104727 [9pp]. DOI:10.1016/j.idairyj.2020.104727.

Weichert S, Jennewein S, Hüfner E, Weiss C, Borkowski J, Putze J, et al. (2013). Bioengineered 2'-fucosyllactose and 3-fucosyllactose inhibit the adhesion of Pseudomonas aeruginosa and enteric pathogens to human intestinal and respiratory cell lines. Nutr Res 33(10):831-8. DOI:10.1016/j.nutres.2013.07.009.

Weichert S, Koromyslova A, Singh BK, Hansman S, Jennewein S, Schroten H, et al. (2016). Structural Basis for Norovirus Inhibition by Human Milk Oligosaccharides. J Virol 90(9):4843-4848. DOI:10.1128/JVI.03223-15.

WHO-AGISAR (2016). *Critically Important Antimicrobials for Human Medicine. 4th Revision 2013*. Geneva, Switzerland: World Health Organization (WHO) / World Health Organization/Advisory Group on Integrated Surveillance of Antimicrobial Resistance (AGISAR). Available at: https://www.who.int/publications/i/item/9789241511469.

WHO (1981). *International Code of Marketing of Breast-Milk Substitutes*. Available at: https://www.who.int/publications/i/item/9241541601.

WHO (2006). *WHO Child Growth Standards: Length/Height-for-age, Weight-for-age, Weight-for-length, Weight-for-height and Body Mass Index-for-age: Methods*. Geneva, Switzerland: World Health Organization (WHO). Available at: https://www.who.int/toolkits/child-growth-standards/standards/weight-for-age. Reviewed In: FSANZ, 2019c.

Wichmann A (2024). Biological effects of combinations of structurally diverse human milk oligosaccharides. Front Pediatr 12:1439612. DOI:10.3389/fped.2024.1439612.

Wu S, Tao N, German JB, Grimm R, Lebrilla CB (2010). Development of an annotated library of neutral human milk oligosaccharides. J Proteome Res 9(8):4138-4151. DOI:10.1021/pr100362f.

Wu S, Grimm R, German JB, Lebrilla CB (2011). Annotation and structural analysis of sialylated human milk oligosaccharides. J Proteome Res 10(2):856-868. DOI:10.1021/pr101006u.

Xu G, Davis JC, Goonatilleke E, Smilowitz JT, German JB, Lebrilla CB (2017). Absolute quantitation of human milk oligosaccharides reveals phenotypic variations during lactation. J Nutr 147(1):117-124 [plus supplementary data]. DOI:10.3945/jn.116.238279.

Yu ZT, Chen C, Kling DE, Liu B, McCoy JM, Merighi M, et al. (2013a). The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. Glycobiology 23(2):169-177. DOI:10.1093/glycob/cws138.

Yu ZT, Chen C, Newburg DS (2013b). Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes. Glycobiology 23(11):1281-1292. DOI:10.1093/glycob/cwt065.

Yu W, Li Y, Liu D, Wang Y, Li J, Du Y, et al. (2023). Evaluation and Mechanistic Investigation of Human Milk Oligosaccharide against SARS-CoV-2. J Agric Food Chem 71(43):16102-16113. DOI:10.1021/acs.jafc.3c04275.

Zabel BE, Gerdes S, Evans KC, Nedveck D, Singles SK, Volk B, et al. (2020). Strain-specific strategies of 2'-fucosyllactose, 3-fucosyllactose, and difucosyllactose assimilation by Bifidobacterium longum subsp. infantis Bi-26 and ATCC 15697. Sci Rep 10(1):15919 [18pp]. DOI:10.1038/s41598-020-72792-z.

ZKBS (2021). Statement of the ZKBS on the suitability of *Escherichia coli* K12-derived strains as part of biological safety measures according to § 8 para. 1 GenTSV. The Central Committee on Biological Safety (Berlin, Germany). Available online: E._coli_K12_as_part_of_biol._safety_measure_2021.pdf